Circadian Timekeeping in the Suprachiasmatic Nucleus: Genes, Neurotransmitters, Neurons, and Astrocytes

11

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Abstract

The nocturnal secretion of melatonin by the pineal gland drives photoperiodic seasonal rhythms in mammals. In turn, the pineal is controlled by daily cues generated intrinsically by the circadian (circa- approximately, *–diem* a day) clock of the hypothalamic suprachiasmatic nucleus (SCN). Photic cues from the retina both synchronize the SCN and also acutely suppress melatonin synthesis. As a result, the duration of the nocturnal secretion of melatonin encodes the length of the night and hence season. This chapter will consider recent developments in understanding how the SCN generates an internal representation of solar time and thereby functions as a clock and calendar. The first level of timekeeping pivots around intracellular transcriptional and translational feedback loops (TTFLs) that constitute cell-autonomous circadian timers. These mechanisms are common to many, if not all, mammalian tissues, but three properties beyond its TTFLs make the SCN the principal pacemaker. First, it is the sole component of the mammalian circadian system to receive retinal input and is therefore directly entrained to the cycle of day and night. The TTFLs of SCN cells are therefore a high-fidelity, internal proxy of solar time. Second, its neural connections to the hypothalamus and beyond enable the SCN to direct rhythmic endocrine (including melatonin) and autonomic and behavioral rhythms that in turn coordinate the innumerable cellular and tissue-based clocks distributed across the body. Third, the

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timekeeping power that enables the SCN to sustain this internal coordination is derived from the circuit-level integration of the ~20,000 neurons into a robust and resilient circuit-level pacemaker. Activity across the cellular TTFLs is tightly synchronized but not simultaneous, as cells peak in activity in different phases. Importantly, this network-level pattern of activity is plastic, the long days of summer increasing the phase dispersal between cells. This response of the SCN to daylength causes a reciprocal widening or narrowing of the dependent melatonin profile. The daily clock thereby generates an internal representation of season. The intercellular mechanisms that underpin network integration are twofold. First, neuropeptidergic cues released from various populations of SCN neurons act in a slow paracrine manner, over circadian time and SCN circuit space, to synchronize and amplify the individual intracellular TTFLs. Second, it has recently become clear that astrocytes play an important role in network function, as they are able to initiate SCN neuronal oscillations and behavioral circadian rhythms and impose their own cell-autonomous period. Analysis of the network-level interactions between neurons and astrocytes will therefore advance understanding of how both circadian and seasonal information is encoded and distributed by the SCN.

Keywords

Circadian · Period · Cryptochrome · Melatonin · Astrocytes · Melanopsin · OPN4

11.1 Introduction: Clocks and Calendars

The cycles of day and night and winter and summer offer particular opportunities and present specific environmental challenges to organisms on a regular, predictable basis. Evolution has therefore favored the development of internal timing mechanisms that allow organisms to anticipate, and thereby prepare for, these events. In vertebrates, the cycle of sleep and wakefulness, which allows adaptive daily alternation between engagement with, and withdrawal from, the world, also imposes a strict temporal order to other systems such as feeding and social engagement. Equally, seasonal rhythms of physiology (fertility), metabolism (adiposity), and behavior (migration) allow species in temperate and polar regions to adapt to their periodically harsh environments, marshaling precious resources to best effect. Such seasonal rhythms are cued by two types of timers, the first is a circannual clock with self-sustained rhythms of approximately 1 year that are entrained (synchronized) by environmental stimuli such as daylength, food availability, and temperature (see chapters by Helm and by West et al. in this issue). When organisms with such a timer are held in experimental isolation, their circannual clocks continue to mark time autonomously and thereby maintain overt seasonal rhythms in the absence of external timing cues. The second mechanism does not involve a circannual oscillator: it responds in an obligate way directly to changes in daylength (photoperiod) to cue seasonal phenotypes. Typically, therefore, when daylength is

experimentally held constant, the organism remains in one or other seasonal state and it does not repeatedly alternate between winter and summer phenotypes. In some larger, long-lived species that are primarily photoperiodic in their seasonality, spontaneous switches between seasonal phenotypes may occur, but they lack the precision of cycles controlled by bona fide circannual oscillators.

In mammals, the location and mechanism of the circannual clock(s) are poorly understood. In contrast, it is very clear that the principal circadian clock (circa-approximately -*diem* day) of the hypothalamic suprachiasmatic nucleus (SCN) plays a pivotal role in photoperiodic time measurement. It does this by controlling the secretion of melatonin by the pineal gland: the long nights of winter are internally represented in the accompanying prolonged nocturnal melatonin profile, whereas in summer, the melatonin profile is truncated. In pinealectomized animals, photoperiodic seasonality is curtailed, but it can be restored by daily delivery of exogenous melatonin with an appropriate winter- or summer-like duration (Bartness et al. 1993). The SCN controls the pineal in two ways, involving both serial and parallel, polysynaptic neural pathways that ultimately regulate the autonomic activation of the secretory pinealocytes. First, it is the conduit for retinally derived signals that acutely curtail the ongoing biosynthesis of melatonin: light at dawn and dusk prevents melatonin synthesis and release. Second, the SCN generates intrinsic circadian signals that initiate (anticipated evening) and terminate (anticipated morning) melatonin synthesis. Thus, when animals are held in continuous darkness, they exhibit a well-defined circadian rhythm of nocturnal melatonin secretion (SCN-dependent), whereas experimental exposure to continuous light suppresses the melatonin signal in a retinally dependent manner. The relative contributions of the acute photic effect and the more sustained circadian control in sculpting the melatonin profile vary between species, with ungulates emphasizing the former and rodents and primates the latter (Lincoln et al. 1985; Stokkan et al. 2007; Maywood et al. 1993; Zawilska et al. 2009). It is nevertheless the case that, by entrainment to the prevailing light/dark cycle, the SCN acts as both a daily clock and a photoperiodic calendar (see the chapter by Sumova and Illnerova), mediating the effects of experienced or anticipated daylight on the neuroendocrine axis (see chapter by Hannibal). Downstream of the pineal, the duration of the melatonin profile is transduced into a seasonal neuroendocrine phenotype both directly and indirectly by melatonin-sensitive cells in the pituitary and hypothalamus (see chapters by Barrett and Morgan; Wood; Dufourny and Franceschini; Stevenson). Although typically thought of in terms of "vegetative" neuroendocrinology, under extreme conditions, these subliminal effects of light, the clock, and melatonin can breakthrough to influence human mood and mental state (see the chapter by Partonen). Given its central role in timekeeping, the purpose of this review is to consider recent developments in understanding how the SCN acts as a central circadian clock, because without it, there would be no photoperiodic calendar in mammals.

11.2 Cell-Autonomous Circadian Timekeeping in Mammals: TTFLs

Before considering the SCN in particular, it is important to review the remarkable discoveries from the past 20 years or so concerning the molecular genetic and cellular bases of circadian timekeeping in mammals. These discoveries came from initial work on *Drosophila melanogaster* and the subsequent tracing of genetic homologies to mammals (Rosbash et al. 2007; Reppert and Weaver 2000), as well as by decoding directly the circadian effects of spontaneous and induced de novo mutations in rodents and more recently in human pedigrees (Takahashi 2016). This body of work has revealed that the circadian clock of mammals is a cell-autonomous mechanism involving intracellular transcriptional/ translational feedback loops (TTFLs). At the heart of the canonical TTFL lies a pair of transcription factors, Clock and Bmal1 (Fig. 11.1). These proteins heterodimerize through their Per-Arnt-Sim (PAS) dimerization domains (two in each protein, PAS-A and PAS-B) and bind to target DNA sequences that contain E-box (Enhancer) regulatory elements via their basic helix-loop-helix DNA-binding motifs. There, the heterodimers recruit the general transcriptional apparatus to drive expression of their target genes, which include *Period* (*Per1 and Per2*) and *Cryptochrome* (*Cry1 and Cry2*). Importantly, these genes encode proteins that also heterodimerize, forming Per/Cry complexes that enter the nucleus and recruit the generic inhibitory machinery to suppress Clock/Bmal1-dependent transcription. After a delay of ~12 h, the Per/Cry complexes have therefore closed a negative feedback loop. De novo Clock/Bmal1-dependent gene expression can only start again once the existing Per and Cry proteins have been degraded, and this in turn takes ~12 h. The delayed negative feedback by Per/Cry is therefore the source of TTFL oscillation, which can be readily monitored by real-time imaging of bioluminescent and fluorescent reporters of Per and Cry transcription and protein abundance (Hastings et al. 2005) (Fig. 11.1).

The aggregate period of the TTFL (~ 24 h) is determined by a suite of factors that incorporates the rates of Clock/Bmal1-dependent transcription, the intracellular dynamics of nuclear transfer, and the stability/degradation of Per and Cry proteins (Reppert and Weaver 2002). Many of these processes are in turn regulated by posttranslational modifications of the various positive- and negative-regulatory proteins. As a result, the period of the cell-autonomous clock can be shortened or lengthened by spontaneous or induced mutations of regulatory enzymes such as protein kinases and ubiquitin ligases that affect the stability of the proteins, or mutations of the core clock proteins themselves that alter their transcriptional activity and/or their susceptibility to such regulation (Hirano et al. 2016; Hastings et al. 2018). Equally, the cell-autonomous clock can be accelerated, slowed down or even stopped by pharmacological targeting of the clock proteins or their regulatory enzymes. In rare human pedigrees, mutations affecting the core clock proteins are associated with inherited sleep disturbances (Jones et al. 2013), whilst the natural range of "lark" and "owl" behavioral phenotypes ("chronotype") seen in human populations is likely a result of multigenic inheritance of several circadian alleles, each of small effect (Roenneberg and Merrow 2016). It is important to note,

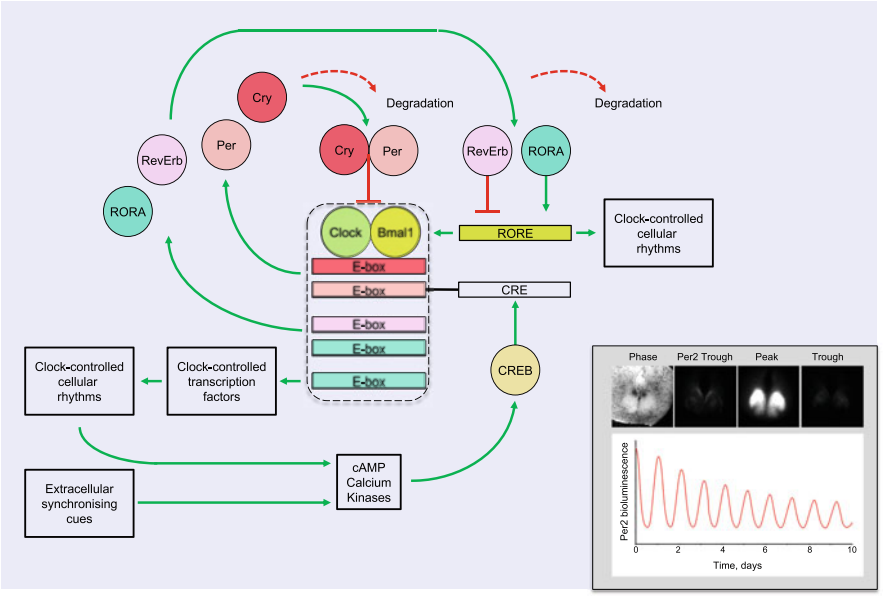

Fig. 11.1 Schematic representation of the interlocked feedback loops of the mammalian circadian clock. At the heart of the circadian oscillator is the control of transcription by the positive regulators Clock and Bmal1, which act via E-box enhancer sequences (dotted line). Genes encoding Per and Cry are activated by Clock/Bmal1, and their protein products in turn oppose the action of Clock/Bmal1 heterodimers, establishing an oscillatory delayed negative feedback loop (the TTFL). Degradation of Per/Cry complexes relieves this negative regulation to initiate a new circadian cycle. The loop is stabilized further by E-box-mediated expression of RevErb and RORA, coregulators of Bmal1 expression, so that TTFL output becomes an input. The circadian signal generated by the TTFL directs cellular functions via a cascade of E-box- and RORE-regulated circadian output genes, with many of them being transcription factors. Additional feedback to the TTFL is provided by cellular cycles of cAMP, calcium and its dependent kinases impinging on CREB, which in turns controls Per expression via calcium/cAMP-regulatory elements (CREs). This pathway is also important for synchronization of the TTFL by extracellular cues. INSET: Upper panel shows phase and bioluminescence images of SCN slice from a Per2::Luciferase mouse, held in static organotypic culture. Bioluminescence images, acquired using a CCD camera, illustrate two serial troughs and one peak of Per2 expression. Lower panel shows aggregate intensity of Per2 bioluminescence signal from the slice recorded over 10 days, exhibiting robust and precise circadian oscillation reflecting the ongoing timekeeping of the TTFL (n.b. declining peak height arises from utilization of luciferin substrate, not attenuation of TFL oscillation) (Hastings, unpublished data)

however, that even though the Per/Cry loop is thought of as the "core" mechanism, it is also sustained by additional accessory feedback loops revolving both around E-boxes and other transcriptional enhancers (e.g. D-Box and CRE-box) (Fig. 11.1). For example, the nuclear hormone receptors RevErbα, RevErβ, and RORA are expressed on a circadian basis via their E-boxes and serve to amplify the core oscillation by controlling the expression of Bmal1 through its RORE regulatory sequences (Preitner et al. 2002) and in their absence, circadian behavior is

compromised (Cho et al. 2012; Yin et al. 2007). Beyond the "core loop", a circadian transcriptional program, driven by clock-controlled transcription factors such as the D-box-binding factors (DBP, TEF, and HLF) (Gachon et al. 2006), as well as RevErb and RORA, transmit and amplify the proxy for solar time developed by the TTFL to coordinate rhythms of cellular metabolism and signaling. In turn, these metabolic outputs of the TTFL feedback to modulate the clock mechanism itself, further stabilizing and amplifying the oscillation (Asher and Schibler 2011). Cell-autonomous circadian timing in mammals is therefore a product of a series of nested and interlocked transcriptional/translational and metabolic feedback loops.

11.3 The SCN as the Principal Circadian Clock and the Hierarchical Organization of Timekeeping in Mammals

The role of the SCN as the central circadian clock of mammals was well established before the discovery of the "clock genes" (Weaver 1998). Anatomical tracing identified it as a retinorecipient hypothalamic nucleus, consisting of approximately 10,000 cells on each side of the third ventricle (Text Box 1). Lesions of the SCN in experimental animals curtailed circadian rhythms of behavior and physiology (including melatonin), identifying it as a necessary component of the circadian system. Equally, compression of the SCN by pituitary tumors in human patients disrupts sleep/wake cycles. Lesions also curtail photoperiodic rhythms in seasonal species as a result of compromised melatonin synthesis. Importantly, when isolated in slice culture, the SCN can exhibit autonomous circadian rhythms of neuronal electrical activity and the dependent release of neurotransmitters and neuropeptides: it is, therefore, a self-sustained "clock in a dish". Moreover, grafting of neonatal SCN to arrhythmic SCN-ablated recipient animals can restore circadian patterning to behavior with a period determined by the intrinsic period of the grafted tissue (Ralph et al. 1990). The SCN is therefore necessary and sufficient for circadian organization of behavior. These observations placed the SCN at a privileged position as one of the three mammalian tissues known to contain autonomous circadian clocks, with the others being the retina and perinatal pineal gland (Patton and Hastings 2018), but the SCN uniquely is able to control circadian behavior and physiology.

Text Box 1: Intrinsically Photoreceptive Retinal Ganglion Cells, Melanopsin, and Circadian Entrainment by Light

In contrast to lower vertebrates, where circadian entrainment to light can be mediated by extra-retinal photoreceptors in the brain and pineal complex, in mammals, the retina is the sole route for photic entrainment of daily and photoperiodic seasonal rhythms (Patton and Hastings 2018). The conventional cone and rod photoreceptors of the outer retina mediate perceptual vision,

(continued)

Text Box 1 (continued)
being hyperpolarized by light of appropriate wavelength and intensity. This leads in turn, via changes in bipolar and amacrine interneuronal signaling, to increased firing of retinal ganglion cells (RGCs) of the inner retina, the axons of which project to the visual relay stations of the brain, including the SCN via the retinohypothalamic tract (RHT). The experimental genetic ablation of rods and cones together renders mice perceptually blind. Such animals can, however, still entrain to cycles of light and darkness: the circadian clock of the SCN does not free run as it would under continuous darkness or following ablation of the eye or the RHT. Assuming the genetic ablations were complete and no residual population of rods and/or cones remained, this observation pointed toward a noncone and nonrod circadian photoreceptor (Freedman et al. 1999). Such receptors also mediate pupillary responses and, of relevance to photoperiodism, suppression of melatonin production by the pineal (Lucas et al. 1999), presumably employing an unknown photopigment. A candidate photopigment, melanopsin (OPN4), was first identified in a screen of G-protein-like receptors from frog skin, which also led to the cloning of melatonin receptors. Mammalian homologues for both receptors were rapidly identified: melatonin receptors being expressed in the SCN and pars tuberalis (Reppert et al. 1994) and hOPN4 in RGCs of the inner retina (Provencio et al. 2000). Did OPN4 mark the unknown photoreceptors of the inner retina, and, if so, was it the photopigment responsible for their responses? Direct evidence to answer the first question was obtained in two ways. First, electrophysiological recordings showed that OPN4-expressing RGCs that project to the SCN are intrinsically photoreceptive (Hattar et al. 2002). Second, calcium imaging of the responses to light of wild-type and rod/cone-deficient retinae in culture revealed an extensive network of light-activated RGCs across the inner retina. These cells exhibited a range of intensity-dependent, sustained, transient, and repetitive responses to light pulses in the absence of rods and cones and expressed OPN4 (Sekaran et al. 2003). The functional necessity of OPN4 for circadian entrainment was demonstrated by triple-mutant mice, lacking rod, cone, and OPN4-mediated phototransduction (Hattar et al. 2003; Panda et al. 2003), which were unable to entrain to lighting cycles, exhibit pupillary responses and suppression of melatonin secretion.

Thus, subliminal, i.e., nonvisual, processing of light information is mediated by a newly discovered class of retinal photoreceptors: intrinsically photoreceptive retinal ganglion cells (iPRGCs) (Lucas 2013). In the case of circadian entrainment, a molecularly defined subpopulation of iPRGCs projects directly to the SCN and alone is sufficient to mediate daily and photoperiodic entrainment (Fernandez et al. 2016). Genetic ablation of these iPRGCs removes nonimage forming vision but leaves intact image formation, which is mediated in parallel via RGCs with input from conventional rods and

(continued)

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cones (Guler et al. 2008). Under natural conditions, however, it is likely that circadian entrainment can be mediated by signaling through OPN4 in combination with rod and cone opsins, rendering OPN4 (but not the iPRGCs expressing it) partially redundant (Van Gelder and Buhr 2016). In contrast to the fast and spatially sharp responses of the conventional rod and cone photoreceptors that mediate conscious vision, the intrinsic responses to light of iPRGCs are characteristically slow, sustained, and spatially diffuse (Schmidt et al. 2011). These properties are better suited to integrate the daily and seasonal changes in daylight, which are relevant to the clock and calendrical functions of the SCN. They are dependent on the cell-autonomous properties of the iPRGCs and also their extensive dendritic morphology but sparse retinal distribution, which allows them to "harvest" photons and so obtain and integrate information on general lighting intensity across visual space.

The discovery of such a novel cell population in the extensively studied retina was remarkable, but equally exciting was the revelation that OPN4 is the molecule that confers photoreceptivity because OPN4 is an invertebrate-like blue-light photopigment. In contrast to rod and cone opsins, which signal via an inhibitory Gi-protein signaling cascade to hyperpolarize the photoreceptor cell, OPN4 acts via a Gq-protein cascade to depolarize the iPRGC and so increase firing rate (Lucas 2013). This mechanism of action of OPN4 is consistent with its structural similarity to invertebrate opsins that signal in a similar way. Thus, light can activate iPRGC firing rate and thereby stimulate the SCN either by the intrinsically induced depolarization or by serial disinhibition conferred by the cone/rod, bipolar cell cascade. Beyond the SCN, it is now also clear that the iPRGCs, through their influence over various subcortical brain area projections to which they connect, can exert a powerful effect on mood and likely cognition (Lazzerini Ospri et al. 2017). Thus, the discovery of the melanopsin-expressing iPRGCs not only revealed a novel anatomical and functional substrate for the subliminal perception and anticipation of light/dark cycles by the circadian system but also highlighted a key pathway connecting seasonal changes to mood control (Fig. 11.2).

This settled view of circadian organization in mammals, with the SCN as the "driving oscillator" causing daily behavioral and physiological rhythms, was, however, radically challenged by the discovery of clock genes, which showed that SCN cells were not the only ones expressing the intracellular timing clockwork. Rather, clock genes are expressed widely across the body, supporting the possibility that other, perhaps many, cells and tissues also contain genetically competent, cell-autonomous circadian oscillators. This was first tested in cell culture using biochemical measures of clock gene expression and then more extensively by developing bioluminescent and fluorescent reporters for long-term real-time imaging of isolated

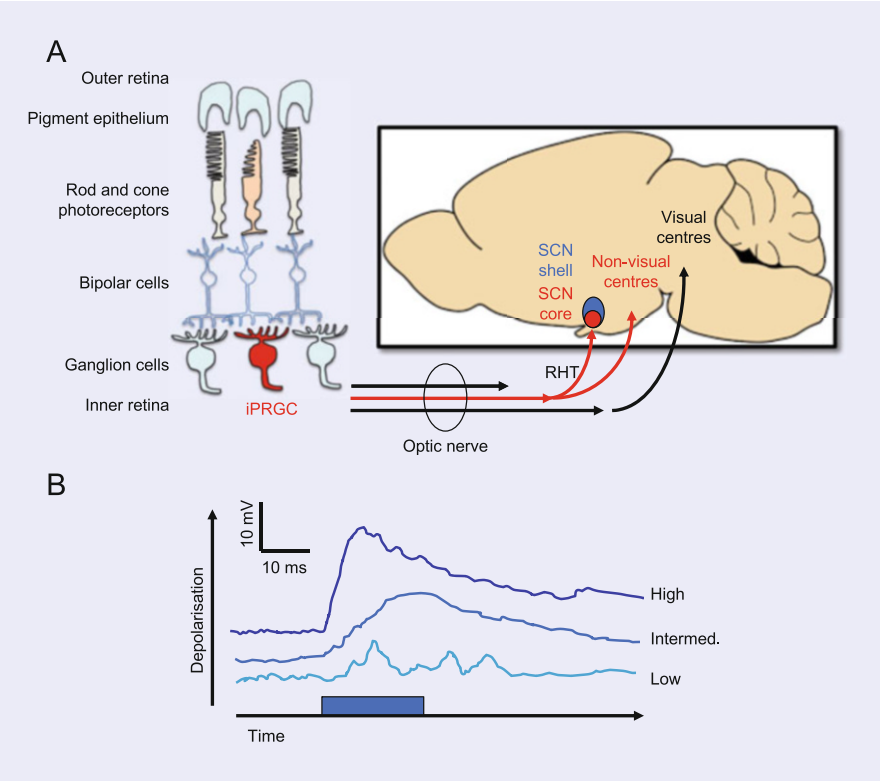

Fig. 11.2 (**a**) Schematic view of the mammalian retina showing location of conventional photoreceptors (rods and cones) in the outer layer connected by bipolar neurons to the retinal ganglion cells (RGCs) of the inner retina. Some of the RGCs (red) express melanopsin/OPN4 and are intrinsically photoreceptive (iPRGCs). RGC axons project to various centers in the brain, the visual centers receiving information from rods and cones. A subpopulation of iPRGCs projects to the SCN core via the retinohypothalamic tract (RHT) to mediate daily and seasonal entrainment and to nonvisual centers to mediate other subliminal responses to light. (**b**) Schematic view of cell-autonomous depolarizing responses of pharmacologically isolated OPN4-positive, iPRGC, to brief light pulses of progressively higher intensity. Note slow, progressive, and sustained, intensity-dependent depolarizing responses. Redrawn from (Berson et al. 2002)

tissues and cell cultures (Nagoshi et al. 2005; Stokkan et al. 2001; Yoo et al. 2004). The resulting discoveries were astonishing: most, if not all, tissues tested were able to express autonomous circadian rhythms of TTFL activity when isolated in culture. There is not a single clock of the SCN: there are numerous clocks active in all major organ systems, all of them pivoted around variants of the intracellular feedback loops driven by Clock/Bmal1, Per/Cry, and associated factors. In turn, these local clocks drive local cellular functions, via the transcriptional cascades and clock-controlled genes noted above, which ultimately determine the behavioral, metabolic, and physiological cycles that adapt mammals to the solar world.

Paradoxically, however, this discovery did not undermine the importance of the SCN; rather, it enhanced it because it revealed that the SCN was not a simple driving pacemaker to which peripheral organs respond passively. Rather, the SCN is capable of orchestrating the intrinsic circadian programs of this astoundingly sophisticated multi-organ system (Hastings et al. 2018) by integrating both the environmental input from the light/dark cycles and the internal feedback by peripheral oscillators. This ensures an accurate representation of internal body time and its corresponding iterations of optimally adapted daily cycles of physiology and behavior. This integration is achieved by daily cues under the control of the neural outputs of the SCN to local hypothalamic nuclei, the brainstem and beyond. These cues, arising from sleep and wakefulness, eating and fasting, and the associated autonomic and neuroendocrine activation, directly and indirectly amplify local tissue-based TTFLs and synchronize them into a coherent whole (Reppert and Weaver 2002; Hastings et al. 2003). In the absence of the SCN, cellular rhythms do not cease, but they do lose amplitude and rapidly desynchronize, leading to the arrhythmia observed at tissue and organismal levels (Yoo et al. 2004; Welsh et al. 2004). The cardinal property of the SCN within this circadian hierarchy is that it can generate autonomously a powerful and coherent rhythm of spontaneous firing rate (SFR) that conveys a set of time cues that are extremely precise and stable (circadian period typically varies by $<1\%$ from cycle to cycle). Indeed, real-time recordings of the expression of Per and Cry at the transcriptional and protein levels in SCN organotypic slice cultures, revealed by various genetically encoded bioluminescent reporters, show that the ensemble TTFL function of the SCN can persist in isolation for months, even years (Hastings et al. 2014). It is this power and robustness (allied to the facts that it is the sole retinorecipient element of the system and that it has suitable efferent pathways to effect internal synchronization), which make the SCN the principal circadian clock.

11.4 The Importance of Neural Circuit-Level Interactions in Circadian Timekeeping by the SCN

When its circuit is intact, either in vivo or as an organotypic slice in culture, the circadian TTFLs of individual SCN cells oscillate with high amplitude and synchronized activity with a shared ensemble period (Fig. 11.1). Peak expression of Per occurs in mid-to-late circadian day, lagging a few hours behind the peak of membrane depolarization and spontaneous electrical firing (Yamaguchi et al. 2003; Brancaccio et al. 2013; Noguchi et al. 2017). In contrast, when circuit-level interactions are compromised, for example, by cellular dissociation, pharmacological blockade of neural activity, or genetic compromise of intercellular signaling, the disconnected cells become sloppy, low amplitude oscillators, losing regularity, definition, and synchrony as they start to oscillate with their cell-intrinsic period. The individual SCN cell in isolation is therefore no more effective as an autonomous oscillator than peripheral cells, such as fibroblasts (Noguchi et al. 2017). The powerful and robust tissue-level oscillation of the SCN is a product of cellular

interactions and is therefore an emergent property of the circuit, which is conferred by intercellular signaling pathways (Hastings et al. 2018). What are the synchronizing cues? Neurons of the SCN all utilize the inhibitory neurotransmitter GABA, but pharmacological blockade of GABA has little effect on the ensemble circadian oscillation of the SCN (but see below in the context of seasonal timing), indicating a minimal role for GABA signaling in determining the emergent properties of the circuit. SCN neurons also express a variety of neuropeptide transmitters, including vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) in the retinorecipient region of the "core" SCN (Text Box 1, Fig. 11.2). The surrounding "shell" of the SCN has limited direct retinal innervation, and cells therein express other neuropeptides, including arginine vasopressin (AVP), prokineticin 2 (Prok2), and their cognate G-protein-coupled receptors (GPCRs) (AVPR1a and 1b, Prokr2) as well as GPCRs for VIP (VPAC2) and GRP (BB2). In terms of circuit coherence, loss of Prok2 or Prokr2 in rodents has little effect on the SCN, but it does compromise circadian behavior, disorganizing evening activity patterns (Prosser et al. 2007). This highlights a role for Prok2 signaling in mediating SCN output. Joint loss of the 1a and 1b receptors for AVP again does not compromise ongoing molecular (TTFL) oscillation and circadian behavior, but in mice lacking these two GPCRs, cellular synchrony across the SCN slice is more readily disorganized by pharmacological perturbations and the circadian behavior of mice is reset much more rapidly by experimentally advancing or delaying the lighting cycle (Yamaguchi et al. 2013). These effects have been interpreted as a role for AVPR1a/1b signaling in binding together the individual clock cells of the SCN shell: it is not necessary for ensemble oscillation to occur, but it does contribute to circuit coherence.

By far, the most dramatic effect of the loss of neuropeptidergic signaling, however, is seen in the case of VIP and its receptor, VPAC2 (Harmar et al. 2002; Colwell et al. 2003). Mice lacking either gene exhibit disorganized circadian behavior, often with elements of very short period, and in slice culture, the SCN rapidly loses ensemble molecular rhythms, as the individual cellular TTFLs damp in amplitude and desynchronize (Maywood et al. 2006). Application of exogenous VIP to a VIP-null SCN acutely resets TTFL amplitude and synchrony, but this is not sustained. When a wild-type SCN is grafted onto a VIP-null slice, however, the graft is able to initiate and sustain very clear TTFL cycles in the mutant "host" SCN (Maywood et al. 2011). Moreover, the period of the cycles is determined by the period of the graft, confirming the graft as the origin of the driving circadian signal. This signal is paracrine: it does not require synaptic contact between host and graft SCN because it can function between slices separated by a porous membrane with a molecular weight cutoff that allows VIP and other small neuropeptides to move through it. Paracrine signaling may be ideally suited to the prolonged timeframe of the circadian cycle and the requirement for cells distributed across the three-dimensional space of the SCN to be smoothly synchronized. Such properties are very different from rapid and locally restricted synaptic signaling. In this paracrine system, the VIP/VPAC2 axis is predominant but not exclusive: in the absence of the VPAC2 receptor, pharmacological blockade reveals additional contributions by

AVP- and GRP-dependent graft-to-host signaling. Furthermore, recent single-cell analyses of peptide and receptor expression suggest that additional neuropeptidergic axes may form an important topological element of SCN circuitry (Park et al. 2016).

Thus, a diversity of neuropeptidergic signals binds together the individual clock cells of the SCN and their TTFLs amplified and synchronized to make the circuit a far more powerful timekeeper than would be the sum of its cellular parts. The likely mechanism underlying this synergy by neuropeptidergic signaling is via GPCR-mediated production of cAMP and mobilization of calcium ($[Ca^{2+}]_i$) from intracellular stores, triggering kinase-dependent transcription of Per genes, via calcium/cAMP-responsive elements (CREs) (Fig. 11.1). Bioluminescence imaging of CRE-dependent transcription has indeed shown CRE-driven transcription in the SCN to be under tight circadian control, peaking in mid-circadian day (CT06, where CT00 is the start of subjective day) (Brancaccio et al. 2013), which coincides with the peaks of cAMP and $[Ca^{2+}]_i$ in the SCN and is suitably a few hours in advance of dependent Per expression, which peaks between CT09-CT12 (Hastings et al. 2014). In addition, rhythms in CRE-mediated transcription are lost in VIP-null SCN, but can be restored by a wild-type SCN graft, consistent with their paracrine regulation, whilst direct chemogenetic activation of the synthesis of cAMP or mobilization of $[Ca^{2+}]_I$, or treatment with exogenous VIP itself, activates CRE-dependent transcription (Brancaccio et al. 2013; Hamnett et al. 2019). Furthermore, pharmacological inhibition of adenylyl cyclase (AC) compromises TTFL function completely, whilst reducing the rate of AC activity slows down SCN circadian period (O'Neill et al. 2008). Complex interactions between intercellular (secreted) cues and intracellular signaling pathways are therefore critical regulators of the core TTFL in the SCN.

The sophistication of this neuropeptide-dependent synchronization is evident from simultaneous imaging of cellular Per expression and $[Ca^{2+}]_i$ levels, both of which are highly circadian and phase-locked (with $[Ca^{2+}]_I$ advanced) on a cell-by-cell basis (Brancaccio et al. 2014). Furthermore, across the SCN, both rhythms show regionally specific phasing, evident as spatiotemporal waves that progress over circadian time from the dorsomedial to the ventrolateral SCN. Although synchronized, the TTFLs of local populations of SCN cells are not, therefore, simultaneously active from region to region; rather, they exhibit stereotypical phase dispersal, with some regions phase-leading others (Fig. 11.3). This generates a spatiotemporal wave that is comparable from slice to slice and independent of overall ensemble period, but its function is not clear (Patton et al. 2016). Theoretical modeling indicates that an oscillatory population is more resilient if phases of synchronized activity are not simultaneous, whilst a biological function may be related to differently phased cells of the SCN conveying temporally distinct cues to their particular targets in the hypothalamus and beyond. Finally, it has been suggested that the phase wave is a mechanism of photoperiodic encoding by the SCN (see below), which raises the question of how light cues from the retina control the cell-autonomous and circuit-level emergent circadian properties of the SCN.

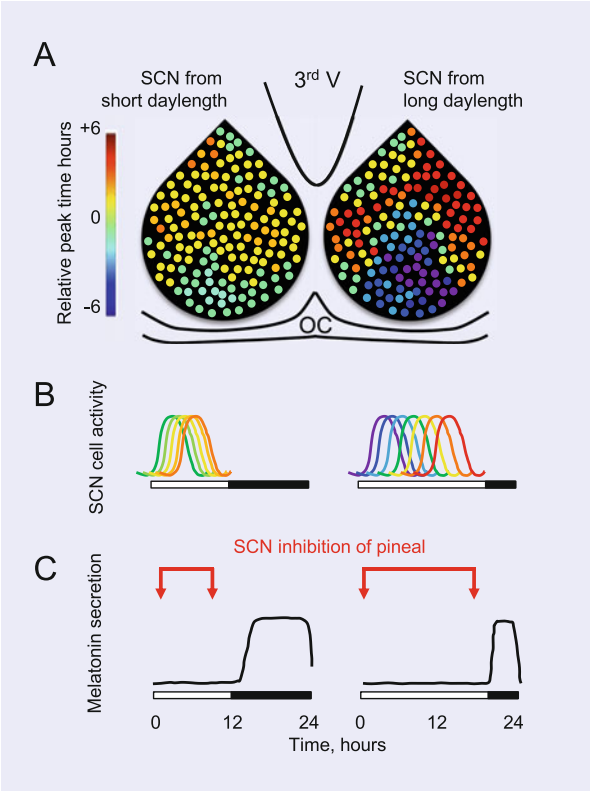

Fig. 11.3 Seasonal (photoperiodic) encoding by phase dispersal of SCN neurons determines profile of nocturnal melatonin secretion. (**a**) Schematic representation of the relative phase distributions of neurons recorded across SCN slices taken from mice previously exposed to a short daylength (12 L::12D, left) or an extremely long day (20 L::4D, right). Circadian phase of each cell is color-coded, dark blue is early and dark red is late. The phase dispersal is much greater in the long daylength and is anatomically explicit with an earlier core and a later shell SCN (based on data presented in (Evans et al. 2013)). (**b**) The photoperiodic phase dispersal distributes active SCN neurons across the circadian daytime, with greater dispersal in long daylengths. (**c**) Under short photoperiods, the compressed phase clustering of SCN inhibitory signaling allows nocturnal pineal melatonin secretion to persist for longer, whereas dispersed SCN cellular activity in summer-like days compresses the melatonin profile. Such changes in the duration of the melatonin profile are the key internal cue driving photoperiodic seasonal rhythms in mammals. OC, optic chiasm, V, ventricle

11.5 Daily and Seasonal Entrainment of the SCN Pacemaker

Entrainment of the SCN is mediated by the light-evoked release of glutamate by terminals of the RHT (Abrahamson and Moore 2001), which are the projections of an anatomically and neurochemically distinct population of retinal ganglion cells

(RGCs) (Text Box 1, Fig. 11.2). Unlike conventional RGCs, those projecting to the SCN are intrinsically photoreceptive due to their expression of the invertebrate-like photopigment melanopsin (Fernandez et al. 2016). Moreover, their responses to light are characterized by slow, sustained spatially diffuse depolarization events, which are better suited to sustain daily and seasonal timekeeping, in contrast to the fast and spatially sharp hyperpolarizing responses of the conventional rod and cone photoreceptors that mediate conscious vision (Schmidt et al. 2011). The VIP cells of the SCN core are the critical gateway for retinal entrainment (Mazuski et al. 2018). By acting through an intracellular, calcium-dependent signaling cascade triggered via NMDA- and AMPA-type ionotropic glutamate receptors, the retinal cues delivered by the RHT terminals activate electrical firing and also induce the expression of Per and other immediate early genes in the core SCN. In the middle of circadian day when spontaneous firing rate (SFR) and Per expression are already high, this stimulation has little additional effect. In contrast, the acute induction of Per transcription at a time when it is spontaneously falling (circadian evening) or is about to rise (circadian morning) resets (delays and advances respectively) the ongoing oscillation of the TTFL in SCN core cells (Shigeyoshi et al. 1997; Herzog et al. 2017). This acute responsiveness of the core SCN to environmental lighting regimes at dawn and/or dusk maintains synchronization of the TTFLs to daily and seasonal time. The next stage of the entrainment response involves second-order effects, whereby VIP and GRP (and perhaps other factors) from activated cells in the core act on cells in the shell that express their cognate GPCRs. As with the circuit-level synchronization of spontaneous cycles in the SCN discussed above, this acute resetting effect of VIP and GRP is mediated by intracellular signaling cascades involving calcium- and cAMP-dependent kinases that converge on CREs to induce Per expression (Hamnett et al. 2019). The same signaling axes are therefore employed both to acutely reset the entire SCN circuit and to sustain its intrinsic coherence and cellular synchrony in the absence of lighting cues.

Under normal conditions, the day-to-day shifts that maintain synchrony of the SCN to environmental light are small, of the order of a few minutes, and thus, they correct the mismatch between the intrinsic circadian period of the animal, which is slightly less than or slightly greater than 24 h, and the exact 24 h period of solar cycles. Experimentally, however, much larger phase shifts of one, even 2 h, can be induced by light pulses presented to mice and hamsters during early or late circadian night (Shigeyoshi et al. 1997; Best et al. 1999). Equally, the SCN slice can be rapidly phase-delayed by 1 h by treatment in vitro with exogenous glutamate (Ding et al. 1994) and VIP (Hamnett et al. 2019) as proxies for activation of the RHT. This sensitivity of the SCN to light at dusk (delays) and dawn (advances) provides the basis not only for daily entrainment but also for photoperiodic encoding by the TTFL. Theoretical considerations have suggested that the SCN circuit incorporates distinct elements that are differentially sensitive to dawn and dusk light, so-called morning (M) and evening (E) oscillators (Daan and Berde 1978), and that as the relative timing of dawn and dusk changes seasonally, then so M and E converge and diverge to create a photoperiodic topology. Whether or not the M and E oscillators are neuroanatomically real and if so, what are their genetic and neurochemical

substrates are unresolved questions (Daan et al. 2001; Hastings 2001). Nevertheless, it is clear that photoperiod alters the waveform of ensemble electrical activity in the SCN (Meijer and Michel 2015), and the discovery of clock genes made it possible to ask how TTFL function in the SCN might react to, and thereby encode, daylength (Inagaki et al. 2007). Initial analyses of Per expression integrated across the entire SCN of mice and hamsters revealed that, similar to the SFR electrical rhythm, the peak of expression is extended under long daylengths, filling most of the light phase under entrained conditions (Messager et al. 1999; Nuesslein-Hildesheim et al. 2000). This extension is retained at least for a few cycles under free-running conditions and therefore reflects a photoperiodically dependent plasticity in the circadian waveform of the TTFL (see Sumova et al. this volume) (Fig. 11.3). Importantly, this change in the diurnal SCN signal duration is the inverse of the nocturnal melatonin duration (the arbiter of photoperiodic rhythms), highlighting its potential physiological relevance. Moreover, it should be noted that even in species that do not exhibit clear photoperiodic seasonal metabolic and physiological traits, not least modern humans and laboratory mice, this seasonal encoding within the SCN waveform, and downstream of it in the pineal, is retained: it is a core property of photoperiodism.

The broader TTFL waveform seen under summer lighting schedules could be a cell-autonomous or a circuit-level effect. Fluorescence and bioluminescence imaging of cellular circadian cycles in SCN slices taken from mice previously exposed to long or short daylengths indicates that it is the latter. Specifically, different cell populations can be seen to peak at different times with the result that the aggregate measure of Per expression is broader but that of individual cells is not (Evans et al. 2013; Yoshikawa et al. 2017) (Fig. 11.3). The "fault-line" in this broadening is between the core and the shell SCN. In slices taken from mice exposed to long photoperiods (20 L:4D), the core peaks ~6 h in advance of the shell, whereas in SCN from mice exposed to 12 L:12D, the shell and core exhibit similar phases. The spatiotemporal wave of the SCN circuit is therefore plastic, responsive to daylength and thus able to incorporate seasonal cues into its circadian oscillation. One cause of this may be that the retinorecipient core is acutely responsive to lighting regimes and rapidly tracks them, whereas resetting of the shell SCN, mediated by core-to-shell neuropeptidergic cues, takes longer and may be less complete. Consequently, under summer photoperiods, the two regions segregate in circadian time, with the core behaving as the putative M and the shell as the E oscillator, broadening the aggregate duration of elevated Per expression and expanding the spatiotemporal wave. Pharmacological studies show that once established, the "stretched", long-day condition is sustained by a combination of VIP and GABA signaling (Evans et al. 2013), but on loss of the photic cues, the system "relaxes" back to a more temporally compressed condition, with VIP promoting synchrony and GABA opposing it to maintain a steady state. Thus, circuit-level rather than cell-autonomous mechanisms are at the heart of the ability of the SCN to encode seasonal time via the circadian TTFL and thereby control melatonin secretion and photoperiodic physiology (Fig. 11.3).

11.6 Neurons, the TTFL, and Circadian Timekeeping in the SCN Circuit

The robust circadian rhythm of SFR suggests that it is principally an output of the SCN TTFL clockwork. But neuronal activity is also necessary for SCN function: pharmacological compromise of action potential firing with tetrodotoxin (TTX) causes rapid damping of the cellular TTFLs and cellular desynchrony, with normal circuit functions being progressively restored when TTX is withdrawn (Yamaguchi et al. 2003). The emergent properties of the circuit; robust oscillation, ensemble period and phase, and cellular synchrony with phase dispersal (i.e. the "wave"), are, therefore, resilient and self-organizing (Hastings et al. 2018). Their control mechanisms are embedded within the structure of the circuit and are expressed via neuronal activity. This importance of neuronal electrical activity to TTFL function occurs at two levels. First, at the cell-autonomous level, electrical firing drives changes in $[Ca^{2+}]_i$ that will in turn trigger the expression of Per, via CREs, and thereby amplify the ongoing TTFL cycle (Fig. 11.1). In this way, an output of the molecular clock, circadian changes in neural firing rates, is also an input to the molecular clock: another example of how the circadian system exploits nested loops to enhance stability and amplitude. Second, at the circuit level, electrical activity will drive the release of neuropeptides, which, as described above, will further bind and amplify the individual cellular TTFLs across the SCN. The powerful effects of coupling between neuronal electrical activity and the cell-autonomous TTFL are therefore a critical feature of the SCN, which are not shared by other, less robust tissue clocks.

An alternative way to explore the relationship between neuronal TTFLs and circuit-level timekeeping is provided by genetic complementation. In Cry-deficient (Cry1, Cry2-null) SCN slices and mice, molecular and behavioral circadian rhythms are absent because the core feedback loop of the TTFL is incomplete. Pan-cellular, transgenic expression of Cry1 or Cry2 under their minimal promoter and delivered by adeno-associated viral (AAV) vectors rapidly initiates SCN TTFL rhythms in slice culture and behavioral rhythms in vivo (Edwards et al. 2016; Maywood et al. 2018). Importantly, the period of these rhythms is determined by the isoform of Cry expressed: Cry1 supports long (>26 h) cycles, whereas Cry2 supports short period (~ 22 h) cycles. This confirms that the AAV-expressed Cry protein is the determinant of rhythmicity and not simply a permissive factor for the expression of a cryptic, Cry-independent oscillator. By making expression of the AAV-Cry1 conditional on a Cre recombinase (Cre), it can be targeted selectively to neurons by cotransfection with an AAV expressing Cre under the neuronally specific synapsin 1 promoter. Under these circumstances, TTFL function in slices and behavioral rhythms in mice are, again, rapidly restored (Brancaccio et al. 2019). Indeed, Cry1-dependent negative regulation of the Per2 bioluminescent reporter in SCN slices is evident within 6–12 h, and full TTFL oscillations are established within 24 h. In vivo it requires approximately 1 week for behavioral rhythms to be initiated, although this may in part reflect the slower timecourse for in vivo transduction. These results showed that when SCN neurons are the only cells in the organism with a molecularly competent

TTFL, they are nevertheless sufficient to drive circadian behavior. Given that the behavioral cycles will govern feeding/fasting and core body temperature rhythms, it is likely (but not yet verified) that such mice will also exhibit peripheral metabolic and physiological circadian rhythms. The ability of these animals to cope effectively with changing lighting regimes and thereby encode photoperiodic cues within their circadian waveform, however, is yet to be tested, although such approaches will be an interesting model to study the relevance of input from the (TTFL-deficient) periphery to the SCN in supporting the flexibility and plasticity required within the SCN for seasonal adaptation. Finally, these results demonstrate that developmental specification and specialization of the molecules and circuitry required to sustain a functional SCN clock, and to control behavioral outputs, occur independently of Cry proteins: mice that have never encountered Cry proteins prior to AAV-mediated complementation have the circuit structures in place to immediately sustain TTFL function and control circadian behavior. Significantly, if AVP signaling in the SCN is curtailed by application of a cocktail of AVPR receptor blockers, the initiation of circuit-wide TTFL function is prevented until washout of the blockers (Edwards et al. 2016). Given that AVP expression is regulated by the TTFL, this suggests that as a cell-autonomous target of AAV-Cry1, AVP is necessary to initiate circuit-wide ensemble oscillations, consistent with role of AVP/AVPR in cellular coupling in the SCN (Yamaguchi et al. 2013).

The contribution of neuronal TTFL function in driving circadian behavior has been explored further by using "translational switching" (Text Box 2, Fig. 11.4) (Maywood et al. 2018). This allows the reversible and temporally explicit expression of Cry1 in SCN neurons but only when a noncanonical amino acid (ncAA) is provided via culture medium. Such acute induction of Cry1 in arrhythmic Cry-null SCN slices rapidly (within 24 h) initiated TTFL function, both cell-autonomously and with circuit-level emergent properties (comparable to those observed with recombinase-restricted neuronal expression) (Fig. 11.5) (Maywood et al. 2018). Importantly, the rhythms dissipated equally rapidly (within 24 h) on withdrawal of ncAA, but were reinstated by repeated addition of ncAAs. These novel manipulations of the cell-autonomous and circuit-level mechanisms governing SCN timekeeping can allow a highly dynamic control of the molecular clockwork: the Cry-dependent TTFL requires little priming to be initiated and has no carryover because Cry-independent oscillations stop after Cry1 expression is terminated. Moreover, treatment of SCN slices with different concentrations of ncAA resulted in dose-dependent expression of Cry1 and consequent dose-dependent lengthening of the period of the TTFL. Higher levels of Cry1 prolonged the phase of negative suppression within the TTFL and thus extended its total duration, with the TTFL period being highly tunable. By providing ncAAs in the drinking water of suitably pretreated Cry-null mice, the same rapid, dynamic, and reversible control was seen at the level of circadian behavior, as Cry1 translation in the SCN was alternately supported and terminated across several weeks *on demand* (Maywood et al. 2018) (Fig. 11.5). Again, transitions between circadian competence and disorder were completed within one or two cycles. It is clear, therefore, that the Cry1-dependent TTFL of SCN neurons is tightly interlocked with electrical firing and that it is pivotal

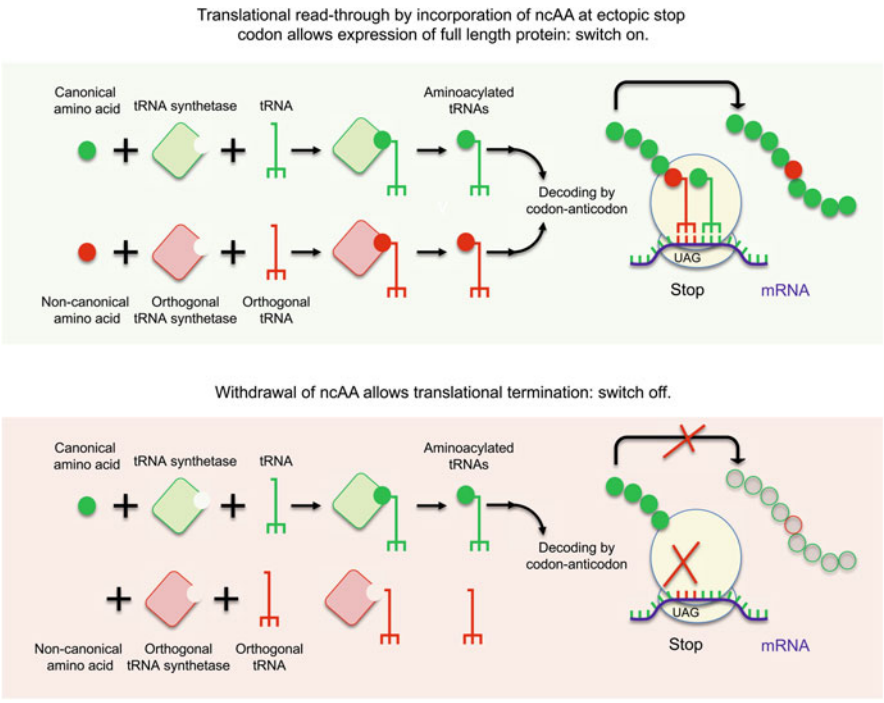

Fig. 11.4 Schematic representation of translational switching by amber suppression. Upper panel: by providing an orthogonal tRNA synthetase to charge an orthogonal tRNA with a noncanonical amino acid (ncAA), an ectopic amber stop codon in the gene of interest can be read through at the ribosome and full-length protein expressed. The protein will incorporate the ncAA at the chosen residue. Lower panel: in the absence of the ncAA, the amber stop codon is free to terminate translation and no full-length protein is expressed (Elements of figure redrawn from (Chin 2017))

in controlling circuit-level and animal-level circadian rhythms, but not in specifying the SCN circuits and extra-SCN neural circuits within which the TTFL and its timing signal operate.

Text Box 2: Amber Suppression for Translational Switching of Protein Expression

The translation of genetic information embedded in mRNA into protein at the ribosome is a central property of living organisms. The unit of translation is the nucleotide triplet codon, which is engaged by a cognate tRNA previously charged with one of the 20 canonical amino acids (AA) used by living organisms. Sequentially, AAs are added to the growing polypeptide chain as specified by the mRNA codon sequence. Translation is terminated when one of the 3 stop codons, which lack a cognate tRNA, is engaged at the ribosome

(continued)

Text Box 2 (continued)
and recognized by a release factor that facilitates ribosomal disassembly. Genetic code expansion is a means for synthetic biology to increase the diversity of amino acids available for protein production by incorporating noncanonical amino acids (ncAA) (Chin 2017). One way to achieve this is to repurpose the mRNA amber stop codon (UAG) to code for ncAA. Artificially engineering amber into a cDNA and thence mRNA encoding a protein of interest provides an avenue for translational incorporation of a ncAA into that protein. This requires the reengineering of a suitable tRNA to recognize amber and be chargeable with the ncAA, alongside a reengineered tRNA synthetase to charge the tRNA with the ncAA (Willis and Chin 2018) (Fig. 11.4).

Suitable orthogonal tRNAs (otRNA) and otRNA synthetases have been developed from prokaryotic factors, and they have been encoded into AAV-based vectors with cell-type specific promoters for use in mouse brain (Ernst et al. 2016). By using synapsin to drive the otRNA synthetase, the potential for translational switching can be restricted to neurons, although access to other cell types is readily achievable (Krogager et al. 2017). To exploit that potential, a second AAV vector is used to deliver the cDNA encoding the protein of interest, bearing an amber stop within the reading frame. Potential expression is again restricted by choice of promoter; in the case of $Cry1^{amber}$, this is the minimal Cry1 promoter sequence capable of conferring circadian transcription (Maywood et al. 2018). To apply the translational switch, SCN slices or the SCN in vivo are transduced with the AAV vector encoding otRNA with otRNA synthetase, alongside the vector encoding Cry1-$Cry1^{amber}$, with a second copy of the otRNA.

There is no expression of Cry1 (evidenced by a C-terminal EGFP tag) in the absence of ncAA, in this case alkyne lysine, as the ectopic amber stop codon terminates translation. Provision of ncAA in culture medium or in drinking water rapidly (within hours) induces protein expression via translational read though of the existing mRNA at the amber stop codon. Equally rapid loss of protein expression follows withdrawal of the ncAA. A critically important aspect of amber suppression is that it does not disregulate the proteome of the transduced cell. Potentially, the effectiveness of endogenous amber codons and correct translational termination could be compromised. This does not, however, appear to be the case (Chin 2017), likely because other aspects such as secondary structure and flanking sequences around the native amber codons maintain their effectiveness, whereas the single ectopic amber codon is more accommodating of translational read through. Applying the translational switch to control Cry1 expression in the context of circadian timekeeping provided proof-of-principle of the utility of the method in the mouse brain, both in vitro and in vivo (Fig. 11.5). The reversibility and dose-dependence of

(continued)

Text Box 2 (continued)
expression offer numerous opportunities to explore the role of other proteins in brain and other functions. Moreover, given the wide chemical diversity of ncAAs, the potential range of novel properties that can be conferred on proteins of interest by incorporation ncAAs has enormous scope for experimental (and potentially therapeutic) applications.

11.7 Are there Pacemaker Neurons in the SCN Circuit?

The neuronal heterogeneity of the SCN, evidenced by the diversity of neuropeptidergic signaling axes noted above (Park et al. 2016), raises the question of whether some neuronal populations/axes are more important than others. Clearly, VIP/VPAC2 signaling is critical for the interrelated functions of cellular synchronization and entrainment by lighting cycles, but beyond that is the SCN, a hierarchical circuit with embedded pacemaker cells, as in the heart for example, or do all cells contribute equally in the computation of its emergent properties? To answer that question, it is necessary to be clear about what "pacemaker" means. Is it a group or "node" of cells that is essential for circuit-wide oscillation to occur or a group that sets the ensemble period of the circuit (literally "makes" its "pace") or that initiates and directs the spatiotemporal waves that progress across the SCN? A series of studies have sought to answer these questions by employing intersectional genetics to manipulate the cell-autonomous properties of defined neuronal groups in the SCN and then test the consequences for circuit function and/or circadian behavior (Hastings et al. 2018). The most extensive test has been of cells expressing Neuromedin S (NMS), which overlap with both VIP and AVP neurons and constitute ~40% of the SCN across core and shell (Lee et al. 2015). Temporary suspension of their synaptic transmission by conditional expression of tetanus toxin light chain reduces the amplitude and cellular synchrony of TTFLs across the SCN slices and reversibly makes mice behaviorally arrhythmic, highlighting the NMS cells as determinants of circadian organization. Furthermore, loss of their cell-autonomous TTFL by conditional deletion of Bmal1 also disrupts circadian behavior, as does over-expression of Per2. Finally, conditional expression of a mutant version of Clock ($Clock^{\Delta 19}$), which lengthens cell-autonomous period, causes reversible lengthening of the period of circadian behavior and the ensemble TTFL of SCN slices. It is clear, therefore, that by the criteria of sustaining circadian oscillation and setting ensemble period, NMS cells act as pacemakers within the SCN circuit. Is this because they have particular distinctive properties?

Paradoxically, the pacemaking roles of NMS cells are not dependent on signaling by their defining neuropeptide because circadian timekeeping is not perturbed in mice devoid of NMS (Lee et al. 2015). This result contrasts dramatically with the role of the VIP/VPAC2 cellular axis in which the neuropeptide and receptor are the

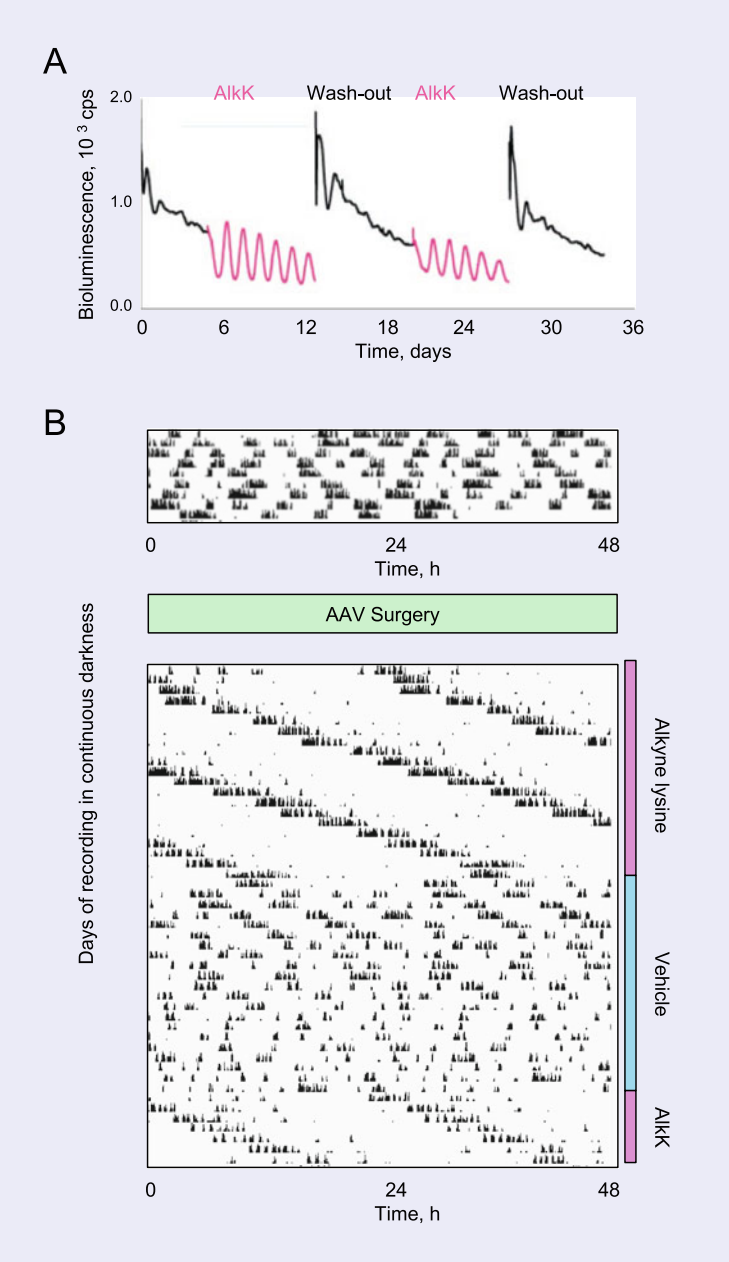

Fig. 11.5 Reversible control of SCN TTFL and behavioral circadian rhythms in Cry-null mice by translational switching of neuronal Cry1 expression. (**a**) Per2::Luc bioluminescence from arrhythmic Cry1,Cry2-null SCN slice transduced with AAVs encoding neuronally specific orthogonal tRNA synthetase, orthogonal tRNA, and $Cry1^{amber}$. Addition of alkyne lysine (AlkK), which will

essential active factors (Harmar et al. 2002; Colwell et al. 2003). It may therefore be the case that any similarly sized population of SCN cells can exert effects comparable to those of the NMS population: circuit functions respond to the quantity of active cells, not their qualitative attributes. Indeed, neurons that express the dopamine 1A receptor (Drd1a), which constitute ~60% of the SCN and also span the core and shell regions of the SCN, can also act as pacemakers (Smyllie et al. 2016). Conditional lengthening of their cell-autonomous TTFL from 20 h to 24 h causes equivalent lengthening of the ensemble period of the SCN and also of circadian behavioral rhythms in mice (Fig. 11.6). This is perhaps unsurprising if these cells are viewed as a dominant majority, but contrary to this expectation, their period-setting role is not fully penetrant insofar as ~30% of such temporally chimeric SCN and mice exhibit an unaltered short period, confirming that control of ensemble period is not simply an effect of numerical advantage. It also shows that the circuit-wide computation is able to incorporate populations of cells with widely divergent cell-autonomous TTFL periods (20 h and 24 h) into a coherent whole with a common ensemble period (20 h or 24 h). In addition, approximately 10% of chimeric SCN and 10% of chimeric mice show circadian rhythms of TTFL activity and behavior that alternate between (Drd1a-determined) long and (nonDrd1a) short periods, revealing an ongoing competition between the two cell populations (nodes) for control of the ensemble period. Moreover, the relative dominance of 24 h Drd1a cells can be redirected by exposure to (antagonistic) 20 h or (facilitative) 24 h lighting regimes, indicating that the circuit-level computation is plastic and sensitive to retinal inputs mediated via the core VIP cells. Notwithstanding this fascinating insight into how circuit-level operations underlie the emergent plasticity, resilience, and robustness of the SCN clock, as with NMS, the Drd1a receptor is not necessary for circadian function per se, although in its absence, the rate of re-entrainment to altered lighting schedules is slower (Grippo et al. 2017). Finally, AVP cells (which partially overlap NMS and Drd1a) can also exert an independent pace-making effect, insofar as genetically slowing their cell-autonomous TTFL or removing the TTFL completely by selective deletion of Bmal1, both lengthen the period of circadian behavioral rhythms in mice (Mieda et al. 2015, 2016). These effects on period are not, however, recapitulated in the SCN slice ex vivo, suggesting that any circuit-level pacemaking effect of AVP cells is less powerful than those of NMS cells and Drd1a cells. But it does leave unanswered the critical question are there neurochemically explicit pacemaking nodes embedded in the SCN circuit?

Fig. 11.5 (continued) allow for translational read through of the amber stop codon and thus sustain neuronal Cry1 expression, immediately initiates negative feedback on Per2 and de novo circadian oscillation. The rhythms are rapidly reversible as the translational switch is turned off and on by removal and addition of AlkK. (**b**) Actograms (double-plotted on 48 h time base for clarity) of arrhythmic Cry1 and Cry2-null mouse before (upper) and after (lower) surgical transduction with AAVs, as above. Provision of AlkK via drinking water rapidly initiates de novo circadian behavior, which is reversibly lost and restored by swapping between vehicle and AlkK (Redrawn from (Maywood et al. 2018))

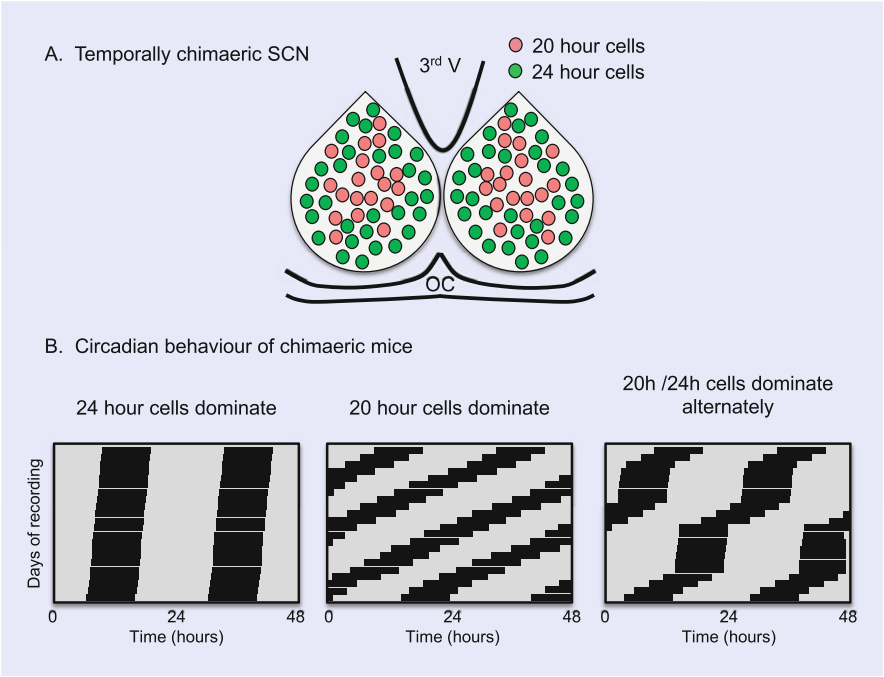

Fig. 11.6 Circadian pacemaking in mice with temporally chimeric SCN reveals network-level interactions between cell groups. (**a**) Schematic representation of temporally chimeric SCN containing cells with cell-autonomous TTFL periods of 24 h or 20 h created by intersectional deletion of the CK1ε^{Tau} alleles in cells expressing Drd1a-driven Cre recombinase. (**b**) Schematic actograms (double-plotted on 48 h time-base for clarity) of mice with temporally chimeric SCN. In the majority of mice (60%), the long period cells predominate (left), but in ca. 30% of mice, the short period cells are pacemakers, whilst in 10% of mice, the two populations control behavioral period alternately (Redrawn from (Smyllie et al. 2016)). OC, optic chiasm; third V, third ventricle

11.8 Neurons Are Not the Only Pacemakers in the SCN Circuit: The Contribution of Astrocytes

Although analysis of SCN timekeeping has focused on neurons, some attention has been paid to their neighboring cells, such as astrocytes, especially in the context of retinal innervation of the SCN. SCN astrocytes strongly express the marker glial fibrillary acidic protein (GFAP), a component of intermediate filaments of the cytoskeleton. Notably, the distribution of GFAP in the SCN is rhythmic, becoming more extensive during circadian daytime and contracting in circadian night (Serviere and Lavialle 1996; Santos et al. 2005). This has been interpreted in the context of astrocytic modulation of retinal innervation to the SCN (closing or allowing access of RHT terminals to SCN core neurons) and/or circadian changes in the metabolic support demanded of astrocytes by neurons, as neuronal electrical firing rates rise

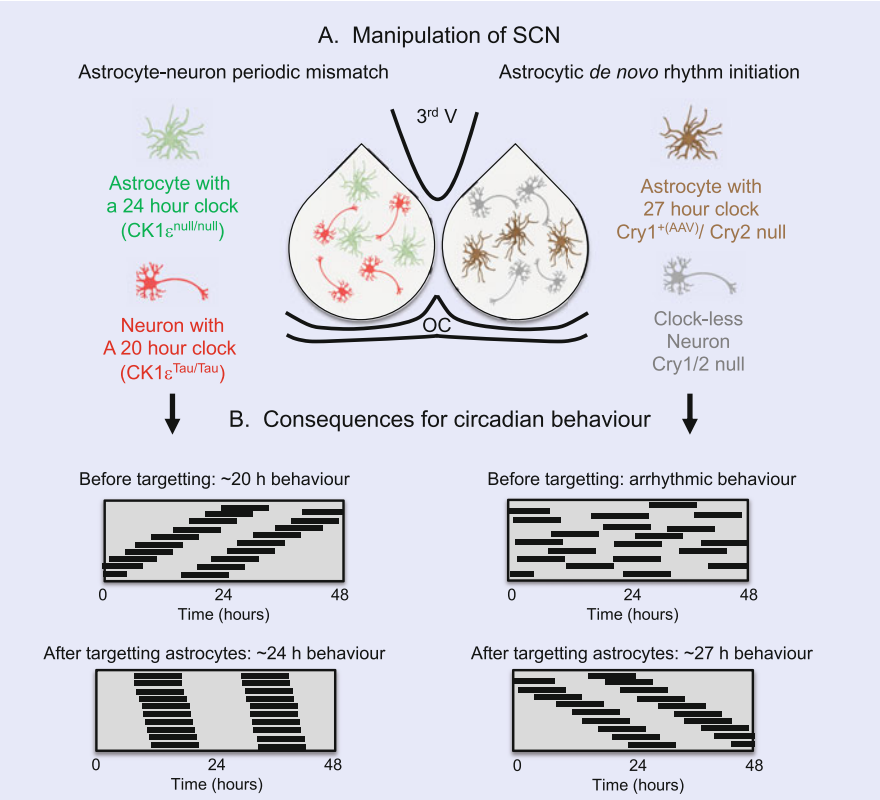

Fig. 11.7 Control of circadian behavior by the cell-autonomous clock of SCN astrocytes. (**a**) Schematic view of intersectional genetic manipulations of SCN neurons and astrocytes. Left: neurons homozygous for $CK1\varepsilon^{Tau}$ have a 20 h cell-intrinsic period, whereas GFAP-dependent deletion of $CK1\varepsilon^{Tau}$ in astrocytes creates an ~ 24 h cell-intrinsic period. Right: in the absence of Cry proteins, SCN neurons lack a functional circadian TTFL, whereas GFAP-dependent expression of Cry1 initiates a long-period oscillation in astrocytes. (**b**) Schematic views of the circadian behavior, under constant darkness, of (left) short period $CK1\varepsilon^{Tau/Tau}$ and (right) arrhythmic Cry1 and Cry2-null mice, before and after AAV-mediated, GFAP-dependent targeting of SCN astrocytes

and fall in circadian time. More recently, a more direct influence of astrocytes on SCN timekeeping has been revealed by conditional manipulation of the astrocytic cell-autonomous TTFL in the SCN. The *Tau* mutant version of casein kinase 1 accelerates the TTFL from 24 to 22 (heterozygous) or 20 h (homozygous). In short-period mutant mice, Cre-mediated deletion of the Tau allele(s) from SCN neurons (and Drd1a neurons, see above) returns behavioral period to wild-type-like 24 h, as would be expected given the central role of SCN neurons in driving behavior. Remarkably, deletion of the mutant allele from SCN astrocytes (targeted by AAV in which the GFAP promoter drives Cre) also causes homozygous mice to change from a 20 h to a 24 h behavioral rhythm (Brancaccio et al. 2017) (Fig. 11.7).

The cell-autonomous TTFL of SCN astrocytes can therefore impose its intrinsic period on the daily behavior of the animal, an effect also seen in heterozygous mice with a period of 22 h that reverts to 24 h when astrocytes are targeted with a different astrocytically specific promoter, Aldh1l1 (Tso et al. 2017). Strikingly, data from this "astrocytic neuronal periodic mismatch" protocol therefore showed that astrocytes of the SCN can function as circadian pacemakers, eliciting circadian patterns of locomotor activity, which are indistinguishable from analogous neuronal manipulations. To test whether or not the astrocytic clock may also be sufficient to initiate de novo SCN and behavioral rhythms, SCN slices from Cry-null mice were cotransduced with AAVs encoding Cre, restricted to neurons, or astrocytes, together with a Cre-dependent Cry1. This protocol of de novo rhythm initiation would reveal the sufficiency of astrocytes in establishing circadian activity. Moreover, because of the absence of competing rhythms in the cell type, which has not undergone the genetic manipulation, it would also allow an easier comparison and contrast of the circadian properties of the newly established rhythms, driven by neurons versus astrocytes. As anticipated, expression of Cry1 in Cry-null SCN neurons initiated circadian TTFL and neuronal $[Ca^{2+}]_i$ rhythms, with a Cry1-definitive long period. Equally, selective expression of Cry1 in astrocytes of Cry-null SCN also initiated TTFL and neuronal $[Ca^{2+}]_i$ rhythms with a Cry1-definitive long period (Brancaccio et al. 2019) (Fig. 11.7). The potency of this control by astrocytes was emphasized further by in vivo studies, in which Cry1 was expressed conditionally in neurons or astrocytes in the SCN of arrhythmic Cry-null mice, which confirmed that Cry1 expression in astrocytes alone was sufficient to both initiate and support long-term daily patterns of locomotor behavior (Brancaccio et al. 2019). Importantly, although the rhythms of the newly generated oscillations were always longer than 24 h, as expected for a Cry1 rescue, astrocytic Cry1 encoded for periodicities that were consistently shorter than those supported by neuronal rescue. Thus, this protocol revealed for the first time that cell-autonomous circadian properties imparted by the astrocytic TTFL clock could be distinguished from those imparted by neurons. Given that astrocytes do not have direct access to behavioral control centers, this also raised the question of how the genetic manipulation of astrocytes could be translated into a motor output, which, although distinguishable in period, was no less robust than the one generated by neurons. Astrocytic Cry1 rescue was capable of reinitiating regular circadian patterns of neuronal $[Ca^{2+}]_i$ in SCN slices showing that the astrocytic TTFL can generate circadian signals able to control cellular circadian function in SCN neurons, which in turn may engage brain centers controlling arousal and locomotion. The possibility to distinguish a cell-autonomous clock in neurons and astrocytes was confirmed by the conditional expression of a bioluminescent transcriptional reporter for Cry*1,* showing that the TTFLs of neurons and astrocytes are indeed differentially phased, with Cry1 peaking at CT11 in neurons and about 6 h later in astrocytes (Brancaccio et al. 2019). This time-of-day-dependent dichotomy in astrocytic and neuronal activities was confirmed by detecting antiphasic $[Ca^{2+}]_i$ rhythms in astrocytes and neurons in wild type SCN slices, which raised the possibility that astrocytes of the SCN would release a signal capable of directly or indirectly inhibiting neuronal activities at nighttime.

11.9 Astrocytically Released Glutamate Synchronizes Activity of SCN Neurons

What type of signal is provided by astrocytes to drive the surrounding neurons? The first indication of such a signal came from real-time recording of extra-cellular levels of glutamate ($[Glu^-]_e$) in SCN slices. By using a genetically encoded fluorescent reporter (iGluSnFR) delivered by AAV vectors, it was possible to show that $[Glu^-]_e$ varied on a pronounced circadian basis, peaking in circadian night in register with astrocytic $[Ca^{2+}]_i$, a phase of neuronal quiescence (Brancaccio et al. 2017). These $[Glu^-]_e$ oscillations were abolished in SCN slices from Cry-null mice, but emerged when Cry1 was expressed in astrocytes. Furthermore, blockers of the astrocytically restricted Cx43 hemichannels (Abudara et al. 2014; Sengiku et al. 2018), which mediate release of glutamate and other "gliotransmitters" into the extracellular space, impaired such newly generated rhythms and also damped rhythms of clock gene expression in wild type SCN. Inhibition of glutamate transporters caused a large rise in $[Glu^-]_e$ as clearance by neurons and astrocytes was curtailed and flattened its circadian pattern. It is even more surprising that this was accompanied by damping and desynchrony of the cellular TTFLs and neuronal $[Ca^{2+}]_i$ rhythms of the SCN, suggesting that the circadian cycle of $[Glu^-]_e$ is important in circuit-level coordination. The regulation of glutamate by astrocytic Cx43 and transporters showed that glutamate is essential to keep circadian oscillations in SCN slices. In this configuration, retinal innervation is absent, and so any spurious effects from glutamatergic RHT stimulation can be ruled out. In the exclusively GABA-ergic and neuropeptidergic SCN circuit, astrocytes may therefore control neuronal activation by releasing gliotransmitters, such as glutamate, to regulate synaptic function (Savtchouk and Volterra 2018). Glutamate can act via metabotropic and ionotropic receptors, with the latter including NMDA- and AMPA-type groups. However, broad-spectrum blockers of NMDARs or AMPARs alone or jointly had no effect on the disruption of SCN timekeeping caused by elevated $[Glu^-]_e$, arguing against an action of glutamate via these molecules. NMDARs are heteromers of NR1 and various NR2 subunits, and NMDARs containing NR1, NR2A, and NR2B mediate the effects of glutamatergic retinal innervation in the core SCN. These variants are sensitive to the broad-spectrum, open channel NMDAR-blocker MK801, but a third NMDA subunit, NR2C, is also expressed in the SCN and NR2C and NR1/NR2C heteromers are not sensitive to MK801. Furthermore, NR2C is expressed in the dorsal SCN shell, not the retinorecipient core, raising the possibility that glutamate acting via NR2C-containing NMDARs may play a previously unrecognized role in SCN timekeeping. Consistent with this idea, treatment of SCN slices with a blocker of NR2C/NR2D complexes caused rapid and reversible damping and period lengthening of the ensemble TTFL. This damping arose from phase-dispersal of the neurons in the NR2C-expressing region of the dorsal SCN and was reflected in the consequent distortion of the spatiotemporal phase wave across the circuit. Given that NR2D is not expressed in the SCN, this effect highlights a role for the NR2C subunits in circuit synchronization. Further evidence comes from electrophysiological studies, showing that NR2C blockade in circadian day (when $[Glu^-]_e$ is minimal)

has no effect on neuronal firing rate or membrane potential, whereas treatment in circadian night, in an environment of high $[Glu^-]_e$ and when SCN neurons are electrically silent, caused an acute and reversible increase in resting membrane potential (depolarization by ~2 mV) and firing rates. These effects were accompanied by an increase in presynaptic $[Ca^{2+}]_i$ levels and decrease in (postsynaptic) cytosolic $[Ca^{2+}]_i$ levels, suggesting that elevated glutamate acts nocturnally on presynaptic NR2C-containing NMDARs to increase the likelihood of GABA release and thereby maintain a suppressive GABAergic tone across the SCN circuit. Removal of this suppressive tone at the beginning of circadian day, when $[Glu^-]_e$ falls as astrocytes become less active, will enable the SCN neurons to increase their spontaneous firing rate and thereby drive their TTFLs at a high amplitude and stable period. How neurons in turn regulate the TTFL of astrocytes is not at all clear, but the working hypothesis is that SCN circuit-level timekeeping arises from interdependent and mutually supportive astrocytic-neuronal signaling. Thus, the "secrets" that make the SCN such a powerful pacemaker are as follows: first, the intracellular linkage between the TTFL and neural electrical activity; second, the intercellular linkage mediated by neuropeptidergic signaling; and third, the mutually supportive antiphasic activities of neuronal and astrocytic TTFLs.

11.10 Perspectives

The SCN is the circadian clock that underpins photoperiodic time measurement in mammals. Understanding of the cell-autonomous basis of circadian timekeeping in the SCN has benefited from advances made in elucidating the composition of the TTFL of other cell types, including cell lines. A new challenge is to understand how this generic TTFL interfaces with the particular electrical and metabolic activity of the SCN neuron and how those neurons are networked together to generate the emergent properties that confer such stability and robustness on the SCN. Calcium-responsive elements have been identified as an access point into the TTFL for cellular signaling pathways, although they are most obvious in *Per1*, and the mechanisms by which other TTFL factors respond to changes in firing rate and/or intercellular signaling await clarification. For example, retinal activation leads to the rapid induction of immediate early genes and their protein products (cFos, Egr1, etc.) in the SCN core. On the other hand, the shell exhibits a low level but sustained circadian oscillation of cFos expression. These acute and tonic changes may in turn direct TTFL gene expression via their AP-1 target sequences. Thus, the conduits for electrical/signaling activation into the TTFL may differ between cell types, for example, between the rapidly resetting core and the slower shell and also between different neuropeptidergic populations. They may also differ between signals that maintain the synchrony of ongoing TTFL oscillations and those that direct phase resetting in response to environmental inputs: are there privileged signaling inputs to the TTFL? Given that TTFLs are active in many other brain regions, lessons learned from the SCN may also be applicable to understand how, for example, cell-autonomous neuronal clocks are integrated and synchronized across the neocortex

or within the hippocampus. Circadian disruption carries a heavy cost for cognition, and loss of local cellular circadian synchrony may be one cause of this.

The nature of the intercellular signals that "bind" the SCN circuit also remains unclear. Notwithstanding the power of neuropeptidergic pathways, the role of GABA transmission in the SCN remains an enigma. It has been suggested that peptidergic and GABAergic cues act in tandem by defining the operational range of membrane potential within which SCN neurons can function (Herzog et al. 2017), an idea prompted by the observation that blockade of GABA signaling can rescue the compromised TTFL of VIP-null SCN, even though it is without effect in wild-type SCN. Thus, the balance between pathways may be as important as the activity in any one particular pathway. In addition, a paradox of the SCN as a GABAergic circuit has been its ability to increase spontaneous firing rate on a circadian basis. Increased firing would increase GABAergic tone across the SCN and thereby hyperpolarize the cells and suppress firing, and so how could peak firing be initiated and sustained for several hours in the middle of circadian day? One possibility is that circadian changes in the reversal potential of the GABAR channels may "toggle" them between inhibitory and excitatory states (Pennartz et al. 2002). Alternatively, the model that astrocytes control GABAergic tone independent of neuronal firing rate, via glutamatergic presynaptic depolarization, resolves the paradox because control of GABAergic tone is now devolved from neurons to astrocytes. Is it the circadian change in astrocytic activity that ultimately directs GABAergic tone and thereby determines neuronal firing rate?

The importance of circuit-level signaling to SCN function raises further questions, such as do some populations of neurons influence the circuit more than others, what is the nature of the neuronal computations performed by the circuit, and what is the logic of its topology as a network? Clearly, the answers to these questions will be mutually informative. Some progress (noted above) has been made in searching for pacemaking cells, but the formal circuit topology of the SCN is not understood. Preliminary data have been used to infer a small-world network (Abel et al. 2016), in which hubs consisting of well connected nodes (cells) are located in the central SCN, whereas the cells in the shell are sparsely connected. The challenge is to determine the biological embodiment of these functionally defined structures. Are the hubs characterized by a particular complement of neurotransmitters, do they have greater influence over ensemble properties, and are there systematic differences in synaptic connectivity between nodes and hubs, should they exist? It is also very likely that the network topology is multiplexed, incorporating levels that operate at different temporal and spatial scales. Whereas the small-world may reflect rapid and local GABAergic synaptic linkages, additional neuropeptidergic paracrine and astrocytic modules may operate orthogonally, at a greater spatial scale, and across a longer time domain.

Calendrical function in the SCN is mediated by temporal (phase) rearrangements between cells, rather than any change intrinsic to the cell-autonomous TTFLs. The identity of the SCN cell populations that encode daylength and the neural pathways by which they direct the pineal gland to generate a photoperiodically appropriate melatonin signal are unknown. Improved understanding of SCN circuit topology

will better inform this problem, but until recently, photoperiodism was envisioned in the context of neurons. The discovery of the circadian role of astrocytes affords new perspectives: could astrocyte-neuronal interactions contribute to the SCN calendar? At a mechanistic level, the ensheathing of RHT synapses by astrocytes seems to change according to time of the day, and so this aspect of the behavior of astrocytes in the SCN core may also change seasonally, tracking daylength. But the more powerful circadian role of astrocytes appears to be in the dorsal SCN, raising the possibility that the roughly defined core-shell schism caused by long photoperiods may be modulated by and/or may involve localized changes in astrocytic functions. For example, once SCN neuronal populations are locked into their long-day state by retinal cues, are astrocytes important for maintaining it?

A final consideration is how the duration of the melatonin signal is read by target cells in the pituitary and hypothalamus. It is clear that the SCN does not make a significant contribution to this process, but that does not mean that circadian factors are not involved. Indeed, the expression of Per1 in the pars tuberalis (PT), a central target of melatonin in all photoperiodic mammals, is dependent on pineal melatonin (Messager et al. 2001) and the amplitude of the peak expression of Per1 mRNA phased to lights on is higher in hamsters held under shorter photoperiods (Messager et al. 1999). A more extensive characterization of circadian gene expression and season in sheep (Lincoln et al. 2002) not only confirmed the broadening of Per expression in the SCN under summer daylengths but also showed that *Cry2* is expressed in the PT at the start of the dark phase. Consequently, its melatonin-dependent phase relationship to the morning-locked Per peak is photoperiodically sensitive, narrowing on winter photoschedules. The downstream mechanisms by which these melatonin-dependent photoperiodic changes in expression of clock genes in the PT generate seasonal phenotypes are poorly mapped. The PT is thought to control prolactin secretion from the anterior pituitary by secreting a yet-to-be-identified releasing factor, whereas seasonal fertility is thought to be controlled by PT-derived TSH acting in the mediobasal hypothalamus to alter local thyroid hormone activity. Given that a large number of genes are photoperiodically regulated in the PT (Dupre et al. 2010), it may be that the cell-autonomous circadian mechanism is not relevant and all the critical timing machinery involves melatonin (Lu et al. 2010). If so, this would extend the functions of canonical clock genes beyond the oscillatory clock and toward a molecular photoperiodic "egg-timer".

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References

Abel JH, Meeker K, Granados-Fuentes D, St. John PC, Wang TJ, Bales BB, Doyle FJ, Herzog ED, Petzold LR (2016) Functional network inference of the suprachiasmatic nucleus. Proc Natl Acad Sci U S A 113:4512–4517

Abrahamson EE, Moore RY (2001) Suprachiasmatic nucleus in the mouse: retinal innervation, intrinsic organization and efferent projections. Brain Res 916:172–191

Abudara V, Bechberger J, Freitas-Andrade M, de Bock M, Wang N, Bultynck G, Naus CC, Leybaert L, Giaume C (2014) The connexin43 mimetic peptide Gap19 inhibits hemichannels without altering gap junctional communication in astrocytes. Front Cell Neurosci 8:306

Asher G, Schibler U (2011) Crosstalk between components of circadian and metabolic cycles in mammals. Cell Metab 13:125–137

Bartness TJ, Powers JB, Hastings MH, Bittman EL, Goldman BD (1993) The timed infusion paradigm for melatonin delivery: what has it taught us about the melatonin signal, its reception and the photoperiodic control of seasonal responses. J Pineal Res 15:161–190

Berson DM, Felice FA, Takao M (2002) Phototransduction by retinal ganglion cells that reset the circadian clock. Science 295:1070–1073

Best JD, Maywood ES, Smith KL, Hastings MH (1999) Rapid resetting of the mammalian circadian clock. J Neurosci 19:828–835

Brancaccio M, Edwards MD, Patton AP, Smyllie NJ, Chesham JE, Maywood ES, Hastings MH (2019) Cell-autonomous clock of astrocytes drives circadian behavior in mammals. Science 363:187–192

Brancaccio M, Enoki R, Mazuski CN, Jones J, Evans JA, Azzi A (2014) Network-mediated encoding of circadian time: the suprachiasmatic nucleus (SCN) from genes to neurons to circuits, and back. J Neurosci 34:15192–15199

Brancaccio M, Maywood ES, Chesham JE, Loudon AS, Hastings MH (2013) A Gq-Ca(2+) axis controls circuit-level encoding of circadian time in the suprachiasmatic nucleus. Neuron 78:714–728

Brancaccio M, Patton AP, Chesham JE, Maywood ES, Hastings MH (2017) Astrocytes control circadian timekeeping in the Suprachiasmatic nucleus via Glutamatergic signaling. Neuron 93 (1420–1435):e5

Chin JW (2017) Expanding and reprogramming the genetic code. Nature 550:53–60

Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT, Chong LW, Ditacchio L, Atkins AR, Glass CK, Liddle C, Auwerx J, Downes M, Panda S, Evans RM (2012) Regulation of circadian behaviour and metabolism by REV-ERB-alpha and REV-ERB-beta. Nature 485:123–127

Colwell CS, Michel S, Itri J, Rodriguez W, Tam J, Lelievre V, Hu Z, Liu X, Waschek JA (2003) Disrupted circadian rhythms in VIP- and PHI-deficient mice. Am J Phys Regul Integr Comp Phys 285:R939–R949

Daan S, Albrecht U, Van der Horst GT, Illnerova H, Roenneberg T, Wehr TA, Schwartz WJ (2001) Assembling a clock for all seasons: are there M and E oscillators in the genes? J Biol Rhythm 16:105–116

Daan S, Berde C (1978) Two coupled oscillators: simulations of the circadian pacemaker in mammalian activity rhythms. J Theor Biol 70:297–313

Ding JM, Chen D, Weber ET, Faiman LE, Rea MA, Gillette MU (1994) Resetting the biological clock: mediation of nocturnal circadian shifts by glutamate and NO. Science 266:1713–1717

Dupre SM, Miedzinska K, Duval CV, Yu L, Goodman RL, Lincoln GA, Davis JR, Mcneilly AS, Burt DD, Loudon AS (2010) Identification of Eya3 and TAC1 as long-day signals in the sheep pituitary. Curr Biol 20:829–835

Edwards MD, Brancaccio M, Chesham JE, Maywood ES, Hastings MH (2016) Rhythmic expression of cryptochrome induces the circadian clock of arrhythmic suprachiasmatic nuclei through arginine vasopressin signaling. Proc Natl Acad Sci U S A 113:2732–2737

Ernst RJ, Krogager TP, Maywood ES, Zanchi R, Beranek V, Elliott TS, Barry NP, Hastings MH, Chin JW (2016) Genetic code expansion in the mouse brain. Nat Chem Biol 12:776–778

Evans JA, Leise TL, Castanon-Cervantes O, Davidson AJ (2013) Dynamic interactions mediated by nonredundant signaling mechanisms couple circadian clock neurons. Neuron 80:973–983

Fernandez DC, Chang YT, Hattar S, Chen SK (2016) Architecture of retinal projections to the central circadian pacemaker. Proc Natl Acad Sci U S A 113:6047–6052

Freedman MS, Lucas RJ, Soni B, Von Schantz M, Munoz M, David-Gray Z, Foster R (1999) Regulation of mammalian circadian behavior by non-rod, non-cone, ocular photoreceptors. Science 284:502–504

Gachon F, Olela FF, Schaad O, Descombes P, Schibler U (2006) The circadian PAR-domain basic leucine zipper transcription factors DBP, TEF, and HLF modulate basal and inducible xenobiotic detoxification. Cell Metab 4:25–36

Grippo RM, Purohit AM, Zhang Q, Zweifel LS, Guler AD (2017) Direct midbrain dopamine input to the Suprachiasmatic nucleus accelerates circadian entrainment. Curr Biol 27(2465–2475):e3

Guler AD, Ecker JL, Lall GS, Haq S, Altimus CM, Liao HW, Barnard AR, Cahill H, Badea TC, Zhao H, Hankins MW, Berson DM, Lucas RJ, Yau KW, Hattar S (2008) Melanopsin cells are the principal conduits for rod-cone input to non-image-forming vision. Nature 453:102–105

Hamnett R, Crosby P, Chesham JE, Hastings MH (2019) Vasoactive intestinal peptide controls the suprachiasmatic circadian clock network via ERK1/2 and DUSP4 signalling. Nat Commun 10:542

Harmar AJ, Marston HM, Shen S, Spratt C, West KM, Sheward WJ, Morrison CF, Dorin JR, Piggins HD, Reubi JC, Kelly JS, Maywood ES, Hastings MH (2002) The VPAC(2) receptor is essential for circadian function in the mouse suprachiasmatic nuclei. Cell 109:497–508

Hastings M (2001) Modeling the molecular calendar. J Biol Rhythm 16:117–123. Discussion 124

Hastings MH, Brancaccio M, Maywood ES (2014) Circadian pacemaking in cells and circuits of the suprachiasmatic nucleus. J Neuroendocrinol 26:2–10

Hastings MH, Maywood ES, Brancaccio M (2018) Generation of circadian rhythms in the suprachiasmatic nucleus. Nat Rev Neurosci 19:453–469

Hastings MH, Reddy AB, Maywood ES (2003) A clockwork web: circadian timing in brain and periphery, in health and disease. Nat Rev Neurosci 4:649–661

Hastings MH, Reddy AB, Mcmahon DG, Maywood ES (2005) Analysis of circadian mechanisms in the suprachiasmatic nucleus by transgenesis and biolistic transfection. Methods Enzymol 393:579–592

Hattar S, Liao HW, Takao M, Berson DM, Yau KW (2002) Melanopsin-containing retinal ganglion cells: architecture, projections, and intrinsic photosensitivity. Science 295:1065–1070

Hattar S, Lucas RJ, Mrosovsky N, Thompson S, Douglas RH, Hankins MW, LEM J, Biel M, Hofmann F, Foster RG, Yau KW (2003) Melanopsin and rod-cone photoreceptive systems account for all major accessory visual functions in mice. Nature 424:76–81

Herzog ED, Hermanstyne T, Smyllie NJ, Hastings MH (2017) Regulating the Suprachiasmatic nucleus (SCN) circadian clockwork: interplay between cell-autonomous and circuit-level mechanisms. Cold Spring Harb Perspect Biol 9

Hirano A, Fu YH, Ptacek LJ (2016) The intricate dance of post-translational modifications in the rhythm of life. Nat Struct Mol Biol 23:1053–1060

Inagaki N, Honma S, Ono D, Tanahashi Y, Honma K (2007) Separate oscillating cell groups in mouse suprachiasmatic nucleus couple photoperiodically to the onset and end of daily activity. Proc Natl Acad Sci U S A 104:7664–7669

Jones CR, Huang AL, Ptacek LJ, Fu YH (2013) Genetic basis of human circadian rhythm disorders. Exp Neurol 243:28–33

Krogager TP, Ernst RJ, Elliott TS, Calo L, Beranek V, Ciabatti E, Spillantini MG, Tripodi M, Hastings MH, Chin JW (2017) Labeling and identifying cell-specific proteomes in the mouse brain. Nat Biotechnol

Lazzerini Ospri L, Prusky G, Hattar S (2017) Mood, the circadian system, and Melanopsin retinal ganglion cells. Annu Rev Neurosci 40:539–556

Lee IT, Chang AS, Manandhar M, Shan Y, Fan J, Izumo M, Ikeda Y, Motoike T, Dixon S, Seinfeld JE, Takahashi JS, Yanagisawa M (2015) Neuromedin s-producing neurons act as essential pacemakers in the suprachiasmatic nucleus to couple clock neurons and dictate circadian rhythms. Neuron 85:1086–1102

Lincoln GA, Ebling FJP, Almeida OFX (1985) Generation of melatonin rhythms. In: Evered D, Clark S (eds) Photoperiodism, melatonin and the pineal gland. Pitman, London

Lincoln G, Messager S, Andersson H, Hazlerigg D (2002) Temporal expression of seven clock genes in the suprachiasmatic nucleus and the pars tuberalis of the sheep: evidence for an internal coincidence timer. Proc Natl Acad Sci U S A 99:13890–13895

Lu W, Meng QJ, Tyler NJ, Stokkan KA, Loudon AS (2010) A circadian clock is not required in an arctic mammal. Curr Biol 20:533–537

Lucas RJ (2013) Mammalian inner retinal photoreception. Curr Biol 23:R125–R133

Lucas RJ, Freedman MS, Munoz M, Garcia-Fernandez JM, Foster RG (1999) Regulation of the mammalian pineal by non-rod, non-cone, ocular photoreceptors. Science 284:505–507

Maywood ES, Chesham JE, O'brien JA, Hastings MH (2011) A diversity of paracrine signals sustains molecular circadian cycling in suprachiasmatic nucleus circuits. Proc Natl Acad Sci U S A 108:14306–14311

Maywood ES, Elliott TS, Patton AP, Krogager TP, Chesham JE, Ernst RJ, Beranek V, Brancaccio M, Chin JW, Hastings MH (2018) Translational switching of Cry1 protein expression confers reversible control of circadian behavior in arrhythmic cry-deficient mice. Proc Natl Acad Sci U S A 115:E12388–E12397

Maywood ES, Hastings MH, Max M, Ampleford E, Menaker M, Loudon AS (1993) Circadian and daily rhythms of melatonin in the blood and pineal gland of free-running and entrained Syrian hamsters. J Endocrinol 136:65–73

Maywood ES, Reddy AB, Wong GK, O'neill JS, O'brien JA, Mcmahon DG, Harmar AJ, Okamura H, Hastings MH (2006) Synchronization and maintenance of timekeeping in suprachiasmatic circadian clock cells by neuropeptidergic signaling. Curr Biol 16:599–605

Mazuski C, Abel JH, Chen SP, Hermanstyne TO, Jones JR, Simon T, Doyle FJ, Herzog ED (2018) Entrainment of circadian rhythms depends on firing rates and neuropeptide release of VIP SCN neurons. Neuron 99(555–563):e5

Meijer JH, Michel S (2015) Neurophysiological analysis of the suprachiasmatic nucleus: a challenge at multiple levels. Methods Enzymol 552:75–102

Messager S, Garabette ML, Hastings MH, Hazlerigg DG (2001) Tissue-specific abolition of Per1 expression in the pars tuberalis by pinealectomy in the Syrian hamster. Neuroreport 12:579–582

Messager S, Ross AW, Barrett P, Morgan PJ (1999) Decoding photoperiodic time through Per1 and ICER gene amplitude. Proc Natl Acad Sci U S A 96:9938–9943

Mieda M, Okamoto H, Sakurai T (2016) Manipulating the cellular circadian period of arginine vasopressin neurons alters the behavioral circadian period. Curr Biol 26:2535–2542

Mieda M, Ono D, Hasegawa E, Okamoto H, Honma K, Honma S, Sakurai T (2015) Cellular clocks in AVP neurons of the SCN are critical for Interneuronal coupling regulating circadian behavior rhythm. Neuron 85:1103–1116

Nagoshi E, Brown SA, Dibner C, Kornmann B, Schibler U (2005) Circadian gene expression in cultured cells. Methods Enzymol 393:543–557

Noguchi T, Leise TL, Kingsbury NJ, Diemer T, Wang LL, Henson MA, Welsh DK (2017) Calcium circadian rhythmicity in the Suprachiasmatic nucleus: cell autonomy and network modulation. eNeuro 4

Nuesslein-Hildesheim B, O'brien JA, Ebling FJ, Maywood ES, Hastings MH (2000) The circadian cycle of mPER clock gene products in the suprachiasmatic nucleus of the siberian hamster encodes both daily and seasonal time. Eur J Neurosci 12:2856–2864

O'Neill JS, Maywood ES, Chesham JE, Takahashi JS, Hastings MH (2008) cAMP-dependent signaling as a core component of the mammalian circadian pacemaker. Science 320:949–953

Panda S, Provencio I, Tu DC, Pires SS, Rollag MD, Castrucci AM, Pletcher MT, Sato TK, Wiltshire T, Andahazy M, Kay SA, Van Gelder RN, Hogenesch JB (2003) Melanopsin is required for non-image-forming photic responses in blind mice. Science 301:525–527

Park J, Zhu H, O'Sullivan S, Ogunnaike BA, Weaver DR, Schwaber JS, Vadigepalli R (2016) Single-cell transcriptional analysis reveals novel neuronal phenotypes and interaction networks involved in the central circadian clock. Front Neurosci 10:481

Patton AP, Chesham JE, Hastings MH (2016) Combined pharmacological and genetic manipulations unlock unprecedented temporal elasticity and reveal phase-specific modulation

of the molecular circadian clock of the mouse Suprachiasmatic nucleus. J Neurosci 36:9326–9341

Patton AP, Hastings MH (2018) The suprachiasmatic nucleus. Curr Biol 28:R816–R822

Pennartz CM, De Jeu MT, Bos NP, Schaap J, Geurtsen AM (2002) Diurnal modulation of pacemaker potentials and calcium current in the mammalian circadian clock. Nature 416:286–290

Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U (2002) The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110:251–260

Prosser HM, Bradley A, Chesham JE, Ebling FJ, Hastings MH, Maywood ES (2007) Prokineticin receptor 2 (Prokr2) is essential for the regulation of circadian behavior by the suprachiasmatic nuclei. Proc Natl Acad Sci U S A 104:648–653

Provencio I, Rodriguez IR, Jiang G, Hayes WP, Moreira EF, Rollag MD (2000) A novel human opsin in the inner retina. J Neurosci 20:600–605

Ralph MR, Foster RG, Davis FC, Menaker M (1990) Transplanted suprachiasmatic nucleus determines circadian period. Science 283:693–695

Reppert SM, Weaver DR (2000) Comparing clockworks: mouse versus fly. J Biol Rhythm 15:357–364

Reppert SM, Weaver DR (2002) Coordination of circadian timing in mammals. Nature 418:935–941

Reppert SM, Weaver DR, Ebisawa T (1994) Cloning and characterization of a mammalian melatonin receptor that mediates reproductive and circadian responses. Neuron 13:1177–1185

Roenneberg T, Merrow M (2016) The circadian clock and human health. Curr Biol 26:R432–R443

Rosbash M, Bradley S, Kadener S, Li Y, Luo W, Menet JS, Nagoshi E, Palm K, Schoer R, Shang Y, Tang CH (2007) Transcriptional feedback and definition of the circadian pacemaker in drosophila and animals. Cold Spring Harb Symp Quant Biol 72:75–83

Santos JWQ, Araujo JF, Cunha MJB, Costa SO, Barbosa ALC, Mesquita JB, Costa MSMO (2005) Circadian variation in GFAP immunoreactivity in the mouse suprachiasmatic nucleus. Biol Rhythm Res 36:141–150

Savtchouk I, Volterra A (2018) Gliotransmission: beyond black-and-white. J Neurosci 38:14–25

Schmidt TM, Do MT, Dacey D, Lucas R, Hattar S, Matynia A (2011) Melanopsin-positive intrinsically photosensitive retinal ganglion cells: from form to function. J Neurosci 31:16094–16101

Sekaran S, Foster RG, Lucas RJ, Hankins MW (2003) Calcium imaging reveals a network of intrinsically light-sensitive inner-retinal neurons. Curr Biol 13:1290–1298

Sengiku A, Ueda M, Kono J, Sano T, Nishikawa N, Kunisue S, Tsujihana K, Liou LS, Kanematsu A, Shimba S, Doi M, Okamura H, Ogawa O, Negoro H (2018) Circadian coordination of ATP release in the urothelium via connexin43 hemichannels. Sci Rep 8:1996

Serviere J, Lavialle M (1996) Astrocytes in the mammalian circadian clock: putative roles. Prog Brain Res 111:57–73

Shigeyoshi Y, Taguchi K, Yamamoto S, Takekida S, Yan L, Tei H, Moriya T, Shibata S, Loros JJ, Dunlap J, Okamura H (1997) Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. Cell 91:1043–1053

Smyllie NJ, Chesham JE, Hamnett R, Maywood ES, Hastings MH (2016) Temporally chimeric mice reveal flexibility of circadian period-setting in the suprachiasmatic nucleus. Proc Natl Acad Sci U S A

Stokkan KA, Van Oort BE, Tyler NJ, Loudon AS (2007) Adaptations for life in the Arctic: evidence that melatonin rhythms in reindeer are not driven by a circadian oscillator but remain acutely sensitive to environmental photoperiod. J Pineal Res 43:289–293

Stokkan KA, Yamazaki S, Tei H, Sakaki Y, Menaker M (2001) Entrainment of the circadian clock in the liver by feeding. Science 291:490–493

Takahashi JS (2016) Molecular architecture of the circadian clock in mammals. In: Sassone-Corsi, P. & Christen, Y. (eds.) A time for metabolism and hormones. Cham (CH)

Tso CF, Simon T, Greenlaw AC, Puri T, Mieda M, Herzog ED (2017) Astrocytes regulate daily rhythms in the Suprachiasmatic nucleus and behavior. Curr Biol 27:1055–1061

Van Gelder RN, Buhr ED (2016) Ocular photoreception for circadian rhythm entrainment in mammals. Annu Rev Vis Sci 2:153–169

Weaver DR (1998) The suprachiasmatic nucleus: a 25-year retrospective. J Biol Rhythm 13:100–112

Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA (2004) Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14:2289–2295

Willis JCW, Chin JW (2018) Mutually orthogonal pyrrolysyl-tRNA synthetase/tRNA pairs. Nat Chem 10:831–837

Yamaguchi S, Isejima H, Matsuo T, Okura R, Yagita K, Kobayashi M, Okamura H (2003) Synchronization of cellular clocks in the suprachiasmatic nucleus. Science 302:1408–1412

Yamaguchi Y, Suzuki T, Mizoro Y, Kori H, Okada K, Chen Y, Fustin JM, Yamazaki F, Mizuguchi N, Zhang J, Dong X, Tsujimoto G, Okuno Y, Doi M, Okamura H (2013) Mice genetically deficient in vasopressin V1a and V1b receptors are resistant to jet lag. Science 342:85–90

Yin L, Wu N, Curtin JC, Qatanani M, Szwergold NR, Reid RA, Waitt GM, Parks DJ, Pearce KH, Wisely GB, Lazar MA (2007) Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways. Science 318:1786–1789

Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS (2004) PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A 101:5339–5346

Yoshikawa T, Inagaki NF, Takagi S, Kuroda S, Yamasaki M, Watanabe M, Honma S, Honma KI (2017) Localization of photoperiod responsive circadian oscillators in the mouse suprachiasmatic nucleus. Sci Rep 7:8210

Zawilska JB, Skene DJ, Arendt J (2009) Physiology and pharmacology of melatonin in relation to biological rhythms. Pharmacol Rep 61:383–410